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# **Regulation and Manipulation of Angiogenic Factors: Impact on Ovarian Function.**

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BSc (Hons)

Doctor of Philosophy  
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## **Declaration**

Except where due acknowledgement is made by reference, the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

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## **Publications and Presentations**

A selection of the work from chapters 3, 4 and 5 has been published in two papers in *Endocrinology*, and they have been included in Appendix 1 of this thesis.

Garside, S.A., Harlow, C.R., *et al.* (2010). “Thrombospondin-1 Inhibits Angiogenesis and Promotes Follicular Atresia in a Novel in Vitro Angiogenesis Assay.” *Endocrinology* **151** (3): 1280-1289

Garside, S.A., Henkin, J., *et al.* (2010). “A Thrombospondin-Mimetic Peptide, ABT-898, Suppresses Angiogenesis and Promotes Follicular Atresia in Pre- and Early-Antral Follicles *in vivo*.” *Endocrinology* **151** (12):

Data obtained during this PhD has also been presented at the following conferences:

British Microcirculation Society Annual conference, London, UK. Poster presentation (April 2008).

Society for Reproduction and Fertility Annual conference, Edinburgh, UK. Poster presentation (June 2008).

Society for Reproduction and Fertility Annual conference, Oxford, UK. Oral presentation prize session (July 2009).

10<sup>th</sup> National Ovarian Workshop, Cambridge, UK. Oral presentation (Sept 2009).

Regulation and Manipulation of Angiogenic Factors: Impact on Ovarian Function  
Society for the Study of Reproduction Annual conference, Milwaukee, USA. Poster  
presentation (August 2010).

## **Abstract**

Angiogenesis is the growth of new blood vessels from existing vasculature; it requires the breakdown of existing blood vessel walls followed by the migration and proliferation of endothelial cells to form the new vessels. It is a complex process that is regulated by many pro- and anti-angiogenic factors and the roles of some of these factors are still unclear. Angiogenesis is a key feature of many pathological conditions including cancer, polycystic ovary syndrome and endometriosis so is an area of great research interest. There are several methods currently available for the study of angiogenesis, both *in vitro* and *in vivo*, and whilst all of these methods have enhanced understanding of angiogenesis, they also have limitations.

The ovary is an excellent model for the study of angiogenesis as it undergoes intense vascular morphogenesis in a cyclical manner. The female reproductive system is unique as no other healthy adult tissue undergoes spontaneous angiogenesis. The tissues in the ovary undergo constant remodelling during both folliculogenesis and the formation and regression of the corpus luteum. Blood vessels are recruited from the ovarian stroma at the preantral stage to form vascular sheaths, in the thecal layer, which surround the developing follicle and supply nutrients, hormones and allow gaseous exchange. As follicular development progresses to the antral stage, when gonadotrophin-dependence is established, increased angiogenesis is essential to sustain development of the rapidly expanding follicle. Previous research into ovarian angiogenesis has focussed on the corpus luteum but the mechanisms of the regulation of angiogenesis during folliculogenesis need further elucidation. The work in this thesis aims to develop and utilise an *in vitro* angiogenesis assay using the culture of

intact preantral and early antral follicles to provide a new approach to the study of follicular angiogenesis. During the course of this thesis this assay was utilised to investigate the effect of various factors on follicular angiogenesis and ovarian function.

The role of the putative anti-angiogenic factor thrombospondin-1 (TSP-1) in the regulation of physiological angiogenesis was investigated using the *in vitro* angiogenesis assay developed during the course of this thesis and the role of TSP-1 in normal ovarian function was investigated using the culture of isolated granulosa cells. The results suggest that TSP-1 is able to inhibit angiogenesis and that it has an extravascular role in the ovary, *in vitro*. These findings were extended to an *in vivo* angiogenesis model where follicular angiogenesis was assessed by quantitative immunohistochemistry for bromodeoxyuridine and the endothelial cell marker CD31. The extravascular role for TSP-1 was also further investigated *in vivo* and was assessed by quantitative immunohistochemistry for activated caspase-3. The results confirmed the findings of the *in vitro* study, indicating that TSP-1 has anti-angiogenic action and acts to clear non-dominant follicles from the ovary through the induction of atresia.

Vascular endothelial growth factor (VEGF) is the main factor involved in stimulating angiogenesis and many advances have been made into elucidating the role, and the mechanisms of action, of VEGF on angiogenesis. Angiopoietin-1 (Ang-1) is considered to be one of the main factors acting in concert with VEGF to stabilise new blood vessels and its role in angiogenesis has been the subject of much

discussion and controversy. This thesis investigates the effects of Ang-1 on follicular angiogenesis and development, using the *in vitro* angiogenesis assay, granulosa cell culture and RNA knockdown experiments. The results have shown that Ang-1 can induce follicular angiogenesis at high doses and that at low doses stimulates pro-survival pathways and inhibits apoptotic mediators.

This thesis describes a novel *in vitro* culture system for the study of angiogenesis in ovarian follicles. Using this system the effects of various factors on follicular angiogenesis and on follicle development and survival have been investigated. Investigations into the mechanisms of action of these factors have also been performed. These studies have improved understanding of the regulation of follicular angiogenesis and have indicated extravascular roles for angiogenic factors in the ovary. Since angiogenesis is a key feature of many pathological conditions, the ability to manipulate angiogenesis and to investigate and quantify the effects of pro- or anti-angiogenic compounds may have important clinical implications.

## Abbreviations

AMH	anti-Müllerian hormone
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
ANOVA	analysis of variance
APAAP	alkaline phosphatase-anti-alkaline-phosphatase
APAF-1	apoptotic protease activating factor-1
bFGF	basic fibroblast growth factor
BMP	bone morphogenic factor
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
CAD	caspase-activated DNase
CAM	chorio-allantoic membrane
CD31	cluster differentiation factor 31
cDNA	complementary deoxyribonucleic acid
CL	corpus luteum
CO <sub>2</sub>	carbon dioxide
COMT	catechol-O-methyltransferase
DAB	3,3' - diaminobenzidine
DISC	death-inducing signalling complex
dH <sub>2</sub> O	deionised water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DMEM	Dulbecco's modified eagle medium

E <sub>2</sub>	estradiol
EBM-2	endothelial basal medium-2
ECM	extracellular matrix
EGF	epidermal growth factor
EG-VEGF	endocrine gland vascular endothelial growth factor
ELISA	enzyme linked immunosorbent assay
ER	estrogen receptor
ERK	extracellular signal related kinase
FADD	Fas-associated protein with death domain
Flk	fetal liver kinase 1
Flt	<i>fms</i> -like tyrosine kinase
Foxo3a	(Forkhead box O3)
FSH	follicle stimulating hormone
g	grams
GDF	growth-differentiation factor
GDF-9	growth differentiation factor-9
GFP	green fluorescent protein
GnRH	gonadotrophin releasing hormone
GPCR	G-protein coupled receptor
h	hour
H&E	haematoxylin and eosin
hCG	human chorionic gonadotrophin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	hypoxia inducible factor



HRP	horse radish peroxidase
HUVEC	human umbilical vein endothelial cell
IAP	integrin-associated protein
ICAD	Inhibitor of CAD
Ig	immunoglobulin
IGF	insulin-like growth factor
IgG	immunoglobulin G
IHC	immunohistochemistry
IL	interleukin
IU	international units
i.v.	intravenous
IVF	<i>in vitro</i> fertilisation
kDa	kilo Dalton
KDR	kinase insert domain receptor
kg	kilograms
KLH	keyhole limpet hemocyanin
LH	luteinising hormone
LIF	leukaemia inhibitory factor
MAPK	mitogen activated protein kinase
2-ME	2-methoxyestradiol
min	minute
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MWt	molecular weight

NBF	neutral buffered formalin
NBT	nitro blue tetrazolium
NDS	normal donkey serum
NGS	normal goat serum
NO	nitric oxide
NRS	normal rabbit serum
OHSS	ovarian hyper-stimulation syndrome
P <sub>4</sub>	progesterone
PBS	phosphate buffered saline
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PECAM-1	platelet endothelial cell adhesion molecule-1
PG	prostaglandin
PGF <sub>2α</sub>	prostaglandin F <sub>2α</sub>
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PVDF-fl	polyvinylidene fluoride-fluorescent
QC	quality control
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate

sec	second
SEM	standard error of the mean
StAR	steroidogenic acute regulatory protein
TBS	tris buffered saline
TGF $\beta$	transforming growth factor $\beta$
Tie	tyrosine kinase with immunoglobulin and epidermal growth factor homology domains
TNF $\alpha$	tumour necrosis factor- $\alpha$
tRNA	transfer ribonucleic acid
TSP-1	thrombospondin-1
TSR	thrombospondin type 1 repeats
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP nick end labelling
UTP	uridine triphosphate
UV	ultra violet
V	volts
v/v	volume for volume
VEGF	vascular endothelial growth factor
VEGFR1	vascular endothelial growth factor receptor 1
VEGFR2	vascular endothelial growth factor receptor 2
VPF	vascular permeability factor
X-gal	5-bromo-4-chloro-3-indolo- $\beta$ -D-galactopyraniside

# **Chapter 1 Introduction**

# **1 Chapter 1 Introduction**

## **1.1 General introduction**

The function of the ovary is to produce fertilisable oocytes and although many follicles begin development only a few will complete the process to become dominant, preovulatory follicles. Follicles are avascular at the primordial stage of development and as a blood supply is necessary for follicle development to occur, angiogenesis must take place to allow the developing follicles to be supplied with gases, nutrients and hormones. Blood vessels are recruited from the ovarian stroma at the preantral stage to form vascular sheaths in the thecal layer, which surround the developing follicle. These new vessels begin to circulate blood to and from the follicle, allowing the exchange of nutrients, hormones and gases (Geva and Jaffe, 2000).

Angiogenesis is the growth of new blood vessels from existing vasculature and it involves the breakdown of existing blood vessels, followed by endothelial cell migration and proliferation and then the stabilisation of the new blood vessels (Folkman and Klagsbrun, 1987). Physiological angiogenesis is rare in the healthy adult, except in the female reproductive system where angiogenesis occurs in a cyclical manner, during follicle development and the formation of the corpus luteum. The majority of angiogenic factors principally act on endothelial cells. However, many of these factors and their receptors are expressed on non-vascular cells so it has been suggested that they may have extravascular effects (Jimenez et al., 2000, Greenaway et al., 2004, Greenaway et al., 2007, Parborell et al., 2008).

This chapter reviews the current understanding of the factors involved in follicular angiogenesis and ovarian follicle development. First the process of follicle development and ovulation is discussed, followed by the regulation of angiogenesis, the clinical relevance of manipulating angiogenesis and the potential extravascular roles of angiogenic factors within the ovary. This chapter serves as an introduction to the following experimental chapters that report studies on the regulation and inhibition of angiogenesis in the ovarian follicle *in vitro* and *in vivo* and on the actions of these factors in non-vascular cells.

## 1.2 Folliculogenesis

Folliculogenesis is the growth of ovarian follicles from the primordial stage to the preovulatory stage of development and is regulated by many endocrine, paracrine and autocrine factors. There are approximately 300,000 to 400,000 primordial follicles present in the human, at birth, (Forabosco et al., 1991, Gougeon et al., 1994, Oktem and Oktay, 2008) but only around 400 will develop into dominant, preovulatory follicles and go on to ovulate (Hillier, 1994) until the cessation of ovulation is reached at the menopause. Follicle development is gonadotrophin-independent at the primordial stage. Follicles become increasingly dependent on gonadotrophins as folliculogenesis progresses and preovulatory follicle development is completely dependent on gonadotrophins (Zelevnik and Hillier, 1996). As only a fraction of follicles that begin development will eventually ovulate, the rest of these follicles will be cleared from the ovary by follicular atresia.

### **1.2.1 Primordial Follicle recruitment**

Follicles are the functional parts of the ovary and as primordial follicles generate all dominant follicles, they are considered the fundamental reproductive units of the ovary. Each primordial follicle consist of a small primary oocyte in meiotic arrest which is surrounded by a single layer of flattened granulosa cells (Sherwood, 2006).

Folliculogenesis begins with the recruitment of quiescent primordial follicles to begin development, and this process is not fully understood. This process has been investigated in many different species including humans, mice, sheep and pigs and this introduction focuses on women where possible, which references to other species as necessary. The initiation of follicle development involves the interaction of many factors including the surrounding follicles, endocrine factors and factors produced by the primordial follicles themselves (Lenton et al., 1984, Gougeon et al., 1994, Spears et al., 1996, Spears et al., 2002). Previous studies have suggested that the oocyte may be responsible for controlling the regulation of follicle recruitment (Lenton et al., 1984, Brankin et al., 2003, Hunter et al., 2005). The initiation of primordial follicle development is regulated by the members of the transforming growth factor (TGF)- $\beta$  superfamily, which include growth-differentiation factors (GDF), activins, inhibins, anti-Müllerian hormone (AMH) and the bone morphogenic factors (BMPs), and are expressed by oocytes and granulosa cells (Fukuda et al., 2000, Oktem and Oktay, 2008). Platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), leukaemia inhibitory factor (LIF), kit ligand and Foxo3a (Forkhead box O3) have also been implicated in regulating the initiation of primordial follicle development (Driancourt et al., 2000, Nilsson et al., 2001, Nilsson

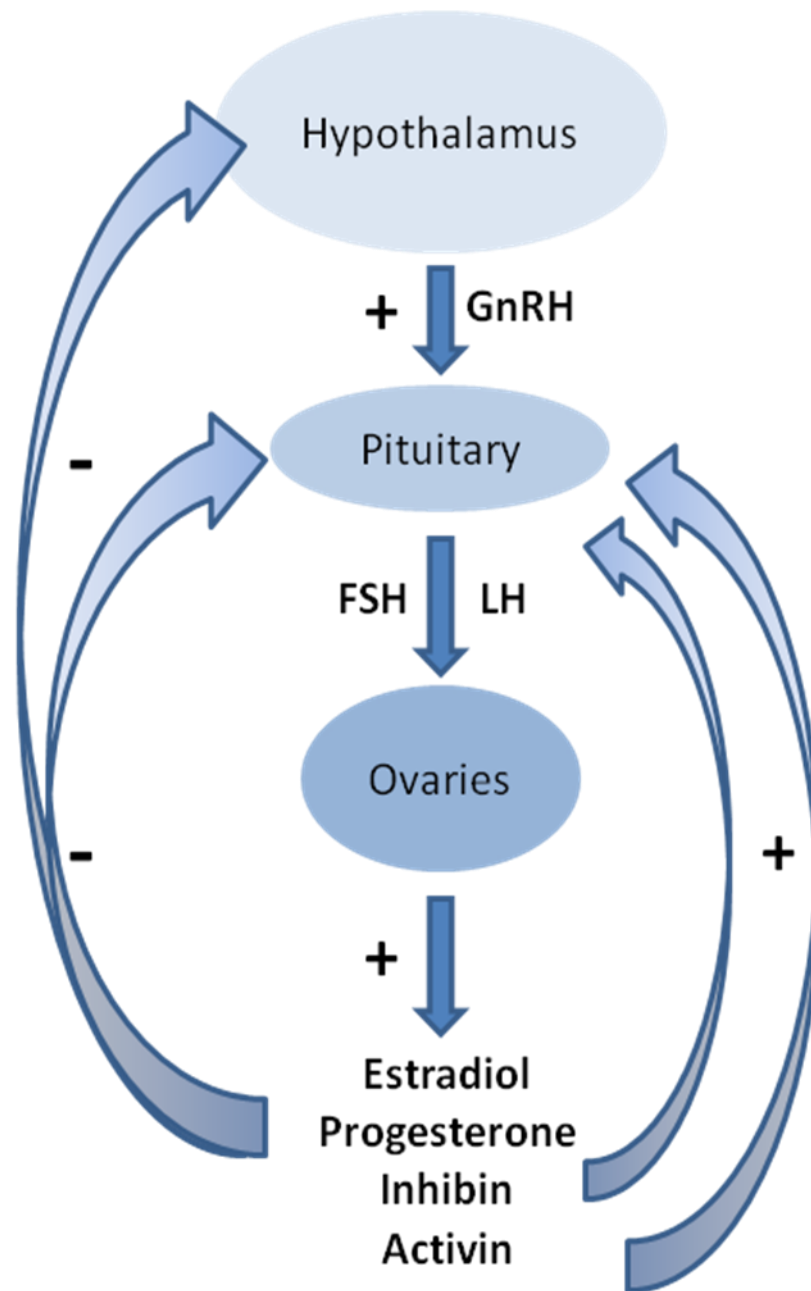
et al., 2002, Smits and Cortvrindt, 2002, Nilsson and Skinner, 2004, Castrillon et al., 2003). This autocrine and paracrine regulation of the recruitment of primordial follicles involves communication between the oocyte and its surrounding flattened granulosa cells but it is thought that the oocyte itself is largely in control of the regulation of follicle recruitment (Lenton et al., 1984, Vanderhyden et al., 1992, Eppig, 2001, Gilchrist et al., 2006, Matzuk et al., 2002, Orisaka et al., 2009). PDGF and bFGF positively regulate follicle activation and increase secretion of kit ligand by pre-granulosa cells. This increase in kit ligand stimulates oocyte growth and therefore promotes the activation of primordial follicles (Tsafriri and Dekel, 1994, Sawetawan et al., 1996). The PI3K pathway, and especially Foxo3a, has been shown to play an important role in regulating the initiation of follicle development (Castrillon et al., 2003). Foxo3a is expressed most highly in the oocytes of primordial follicles and is swiftly down-regulated in the oocytes of larger follicle, supporting the suggestion that Foxo3a is a crucial suppressor of follicle activation and oocyte growth (Liu et al., 2007, Reddy et al., 2010). A pivotal study by Castrillon *et al* (Castrillon et al., 2003) with Foxo3a<sup>-/-</sup> mice showed that the absence of Foxo3a leads to over-abundant activation of primordial follicles, resulting in premature ovarian failure and infertility. Foxo3a has been shown to act as an inhibitor of follicle activation and the down-regulation of Foxo3a in oocytes has been suggested to be essential for the initiation of oocyte growth (Castrillon et al., 2003, Liu et al., 2007, Adhikari and Liu, 2009). Therefore Foxo3a is thought to be essential in negatively regulating the activation of follicle development.



Along with promoting their own activation, primordial follicles release an inhibitory signal that negatively regulates the activation of other primordial follicles (Gougeon and Chainy, 1987, Gougeon et al., 1994). AMH is a dimeric glycoprotein that is produced by granulosa cells of developing follicles (Rajpert-De Meyts et al., 1999, McGee et al., 2001, Durlinger et al., 2002, Drummond, 2005) and it negatively regulates the recruitment of adjacent follicles (Young and Jaffe, 1976, McNatty et al., 1979). AMH also decreases the sensitivity of larger follicles to follicle stimulating hormone (FSH) which reduces the recruitment of follicles (Durlinger et al., 2001, Hillier, 2009).

### **1.2.2 Gonadotrophin production**

The synthesis and release of pituitary gonadotrophins is controlled by the binding of gonadotrophin-releasing hormone (GnRH) to its receptor on anterior pituitary gonadotrope cells and the activation of protein kinase C (PKC). The gonadotrophins FSH and luteinising hormone (LH) are heterodimeric polypeptides that are made up of a common  $\alpha$ -subunit and a unique  $\beta$ -subunit that confers biological specificity. GnRH is released in a pulsatile manner from the GnRH neurones in the hypothalamus (Clarke, 1996). After it is released by the hypothalamus, GnRH travels to the anterior pituitary, binds to the GnRH receptor and stimulates gonadotrophin synthesis and release. Binding of GnRH to its receptor triggers a rapid increase in intracellular calcium levels and activation of PKC, both of which may lead to enhanced synthesis of gonadotrophin subunits. In addition, gonadal hormones, such as oestrogen, progesterone and inhibin, can regulate the action of GnRH (Clarke, 1996).



**Figure 1.1 The hypothalamic-pituitary axis**

*GnRH from the hypothalamus stimulates the secretion of pituitary gonadotrophins, LH and FSH, which directly regulate ovarian function. Ovaries exert negative feedback on the pituitary via oestrogen, progesterone and the inhibins and activins.*

### **1.2.3 Follicle development**

Follicle development begins with the transition of primordial follicles to primary follicles. The flattened, epithelial-like granulosa cells that surround the primordial follicle expand and become cuboidal in appearance and as the follicle gains a single layer of cuboidal granulosa cells it becomes a primary follicle. At this stage follicles begin to express the FSH receptor on their granulosa cells and oocyte growth and differentiation is initiated (Hillier et al., 1980). The initial stages of follicle growth are independent of gonadotrophins; however FSH is required for the development of follicles to the preantral stage (Maruo et al., 1993).

Once primordial follicles are recruited into the growing follicle pool the paracrine regulation of oocytes and granulosa cells become important for follicle development (Gilchrist et al., 2006). The development of follicles from the primary to the preantral stage is dependent on paracrine signalling by members of the TGF $\beta$  superfamily. Activins, along with GDF-9, BMP-15 and BMP-6, are known to increase granulosa cell proliferation and to modulate the sensitivity of granulosa cells to FSH (Hillier, 2009). TGF $\beta$  can also induce expression of the FSH and LH receptors and increase production of inhibin (Knight and Glistner, 2006, Hillier, 2009). Inhibins stimulate the production of androgens in thecal cells and are important in regulating the LH-dependent stages of follicle development (Hillier, 2009). Small antral follicles produce more activin than inhibin whereas larger follicles produce more inhibin (Knight and Glistner, 2006). The increased levels of inhibin expression in larger follicles results in increased androgen production, thus

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sustaining a high level of estradiol secretion and suppressed FSH production (Knight and Glister, 2001, Spears et al., 2002, Knight and Glister, 2003).

In addition to the paracrine regulation of follicle development, the members of the TGF $\beta$  superfamily also regulate follicle development through autocrine signalling. For example GDF-9, which is produced by the oocyte, can act on the oocyte through autocrine actions to regulate its own development, and when GDF-9 is absent it results in abnormal oocyte growth, giving evidence for autocrine actions of GDF-9 on the oocyte (Dong et al., 1996). An autocrine role for inhibin has been suggested as it is produced by granulosa cells and the binding site for inhibin- $\alpha$  is present on granulosa cells (Findlay, 1993). It has also been suggested that inhibin can inhibit estradiol production in rat granulosa cells and that it may inhibit FSH-induced aromatase activity in granulosa cells by autocrine action. Another member of the TGF $\beta$  superfamily, activin, could also have an autocrine role in granulosa cells as the  $\beta$ -subunit has been shown to be expressed on granulosa cells. Activin promotes granulosa cell proliferation and can up-regulate the expression of LH receptors on granulosa cells (Chedrese et al., 2009).

The gonadotrophins and members of the TGF $\beta$  superfamily are vitally important in regulating follicular development, often by endocrine, autocrine and paracrine signalling. In addition to this, these molecules are involved in the regulation of granulosa cell survival. Several of these factors, including GDF-9 and activin have been shown to stimulate the pro-survival PI3K/Akt pathway (Wang and Tsang, 2007) which is also stimulated by several angiogenic factors, through either

autocrine or paracrine signalling. Several angiogenic factors, such as thrombospondin-1 (TSP-1) (Lawler et al., 1998, Hugo and Daniel, 2009), can regulate members of the TGF $\beta$  superfamily, suggesting that they have the potential to influence granulosa cell function. Therefore the effect of angiogenic factors on granulosa cell function will be investigated during the course of this thesis.

#### **1.2.4 Antral follicle development**

As the preantral stage of development progresses the oocyte increases in size and develops its zona pellucida. Granulosa cells proliferate and accumulate to form multiple layers around the oocyte. Cells are then recruited from the ovarian stroma to form the two layers of the theca; the theca interna that can differentiate into interstitial cells, and the theca externa that can differentiate into smooth muscle cells (Fortune and Eppig, 1979, Gougeon, 1996, Braw-Tal and Yossefi, 1997, Lundy et al., 1999, Smits and Cortvrindt, 2002). A distinct thecal layer is evident at different stages of folliculogenesis in different species. In rodents, it can be seen as early as the late primary/early preantral stage of follicle development (Fortune and Eppig, 1979), but cannot be seen in the primate, bovine or ovine follicle until the mid or late preantral stage of follicular development (Gougeon, 1996, Braw-Tal and Yossefi, 1997, Lundy et al., 1999). The development of the thecal layer is accompanied by the initiation of angiogenesis in the preantral follicles which allows blood to circulate and to bring nutrients and gonadotrophins to, and waste and secretory products from, the developing follicles.

The transition of follicles from the preantral to the antral stage is characterised by the development of a fluid filled cavity on one side of the oocyte that contains follicular fluid (Hillier, 1991). The antrum begins to form after 6-7 layers of granulosa cells have surrounded the follicle and granulosa cells surrounding the oocyte form the cumulus layer (Gougeon, 1996). At this stage of follicular development the early antral follicles are responsive to FSH but not to LH and LH receptors are not present on granulosa cells (Zelevnik et al., 1981, Smitz and Cortvrindt, 2002). Antral follicles are present at all stages of the human ovarian cycle, due to primordial follicles continuously entering the pool of developing follicles, but only early antral follicles with sufficient FSH receptors will be selected for dominance and potentially go on to ovulate as the maturation of early antral follicles is regulated by FSH (Baker and Spears, 1999). FSH binds to the FSH receptors expressed on granulosa cells, which activates adenylyl cyclase and cAMP-dependent protein kinases, leading to the expression or inhibition of various genes involved in cell development (Richards, 1994). Tonic stimulation by FSH is sufficient to maintain the development of early antral follicles, however cyclic levels of FSH are required for further follicular development.

### **1.2.5 Selection of dominant follicle(s)**

Follicular development progresses as the level of FSH expression increases and antral follicles enter preovulatory development, following the withdrawal of the negative feedback action of the estradiol, progesterone and inhibin produced by the corpus luteum of the previous cycle (Goodman and Hodgen, 1983, Baird et al., 1984). In humans and other mono-ovulatory species only one follicle is commonly

selected for dominance whereas in other species, multiple follicles are selected for dominance which is reflected in the multiple births of multi-ovulatory species. It is thought that sensitivity to FSH is one of the determining factors in follicle dominance (Zelevnik and Kubik, 1986) as larger, more dominant follicles have greater levels of FSH receptor expression on the granulosa cells whilst less mature follicles have fewer FSH receptors. To continue to develop, follicles need a 'threshold' level of FSH stimulation and follicles that do not get this level of FSH stimulation are not chosen to begin preovulatory development. In addition, FSH stimulates the synthesis of inhibin by granulosa cells (Findlay, 1993), which suppresses secretion of FSH by the pituitary (Groome et al., 1995). As FSH is required for follicle development to progress, this decline in the concentration of FSH prevents the further development of other antral follicles and they undergo atresia (Baker et al., 2001).

Once follicles enter preovulatory development they begin to produce oestrogen and the follicle(s) that produce the highest levels of oestrogen are selected for dominance (Goodman et al., 1977, Zelevnik et al., 1981). The production of oestrogen by the dominant follicle suppresses FSH secretion by negative feedback at the hypothalamic-pituitary axis (Spears et al., 2002). In the follicular phase of the cycle the plasma FSH level rises and P450<sub>arom</sub>, which is responsible for oestrogen synthesis, is increasingly expressed in the granulosa cell layer of the dominant follicle(s). Both granulosa and thecal cells, regulated by FSH and LH, are crucial to oestrogen synthesis. LH stimulates the production of testosterone and androstenedione by thecal cells, but thecal cells cannot aromatise C<sub>19</sub> androgens to oestrogens (Hillier et al., 1981). Granulosa cells lack the P450<sub>C17</sub> enzymes that are

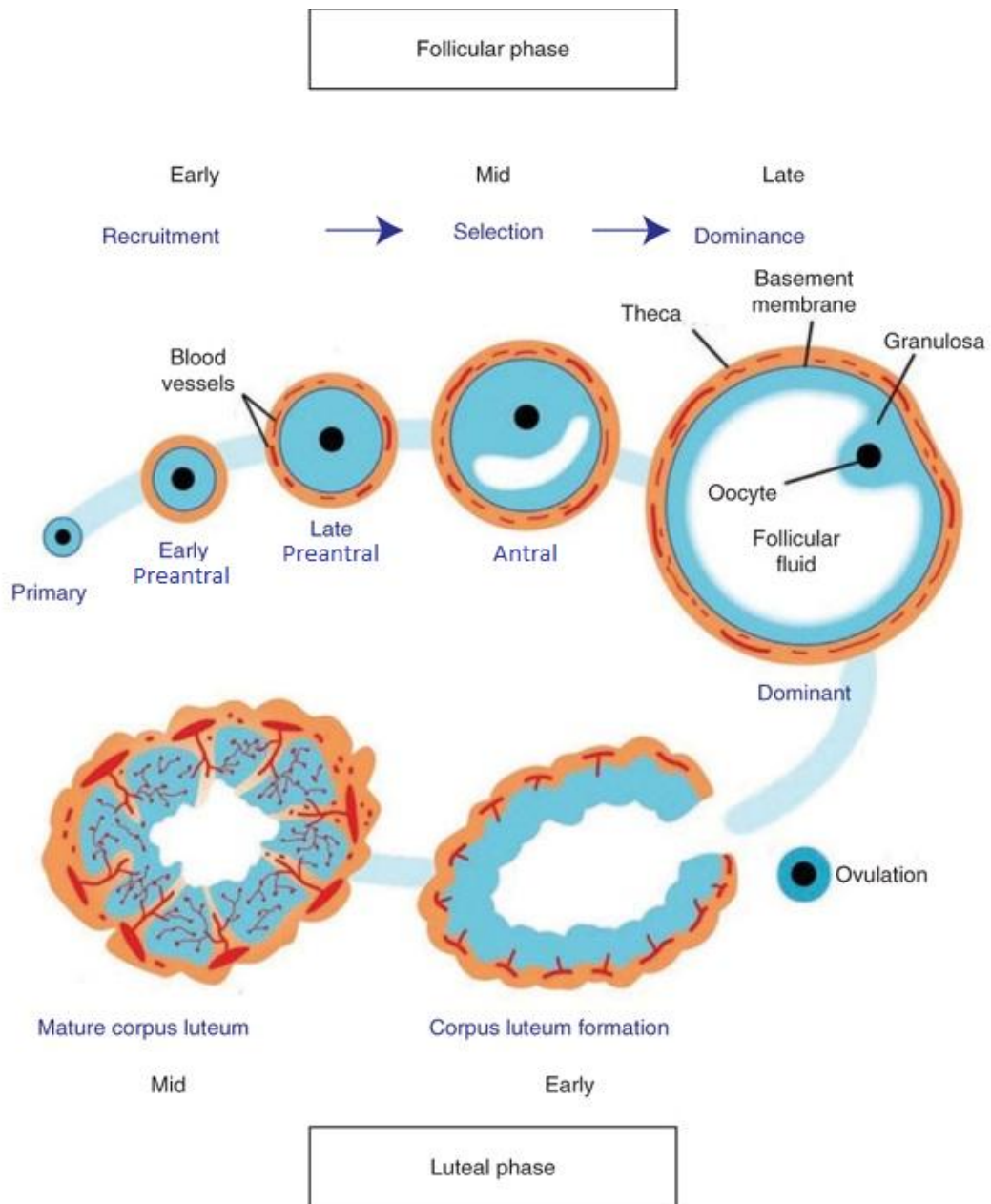
required to metabolise steroids to androgens but, in contrast to thecal cells, they are stimulated by FSH to acquire the aromatase enzymes that convert androgens to oestrogens. The thecal layer is highly vascularised so is directly supplied with steroids, and thecal cells contain the steroidogenic enzymes necessary to synthesise androgens from acetate and cholesterol. The vasculature in the thecal layer is well developed in preovulatory follicles so the granulosa cells in preovulatory follicles are well placed to respond to changes in the level of circulating LH, which is important in the maintenance of follicular dominance.

It is thought that the presence of LH receptors on granulosa cells of the dominant follicle(s) protect it from the decline in FSH levels (Yong et al., 1992, Baker and Spears, 1999), as both FSH and LH act via the stimulation of adenylyl cyclase and an increase in intracellular cAMP (Gonzalez-Robayna et al., 2000). The number of follicles that secrete enough oestrogen to become dominant in a cycle varies from species to species (Goodman et al., 1977, Zeleznik et al., 1981) and they are selected from a cohort of healthy antral follicles in the ovaries (Gougeon and Lefevre, 1983).

Ovulation is the culmination of follicular development and is triggered by the LH surge from the anterior pituitary (van den Hurk and Zhao, 2005). This LH surge is triggered by the high levels of estradiol produced by the dominant follicle(s). The LH surge reactivates maturation of the oocyte and also stimulates steroidogenic cells to induce an inflammatory response which prepares the preovulatory follicle for rupture (Espey, 1980). After the LH surge occurs, the follicle wall ruptures and the oocyte is released from the follicle (Balasch and Fabregues, 2002). An area of the preovulatory



follicle wall weakens as ovulation approaches and at ovulation the apex of the follicle ruptures, releasing the cumulus-oocyte complex. After ovulation the granulosa cells in the preovulatory follicle undergo luteinisation and the corpus luteum begins to form.



**Figure 1.2 Diagram of follicular development**

Diagram illustrating the stages of follicular growth, ovulation, and corpora lutea formation and maturation. Blood vessels, shown in red, appear within follicles at the preantral stage. They are confined to the thecal layer by the presence of a basement membrane. At ovulation, the basement membrane breaks down and, in association with intense angiogenesis, the blood vessels invade the resulting corpora lutea. (Adapted from Fraser 2009).

### **1.2.6 Follicular atresia**

Although many follicles are recruited to begin development only a few ever develop to the preovulatory stage and go on to ovulate. In fact, 99% of all follicles undergo atresia and die. The majority of follicular atresia occurs during foetal development and it is controlled by a balance of pro-survival factors and pro-apoptotic factors (Shikone et al., 1996), including gonadotrophins and members of the TGF $\beta$  superfamily (Gosden and Spears, 1997). Follicular atresia begins in the granulosa cells before progressing to the thecal cells. In fact, the apoptosis of granulosa cells is one of the main ways that follicles are cleared from the ovary. There are many factors involved in regulating follicular atresia including the caspase cascade, the Bcl-2 family and mitogen-activated protein kinases (MAPK) signalling (Markstrom et al., 2002). The Bcl-2 family consists of both pro-apoptotic and anti-apoptotic factors. The pro-apoptotic members of the Bcl-2 family such as Bax stimulate cytochrome C which then goes on to trigger the caspase cascade, which is a well known inducer of apoptosis. Additionally, the MAPKs play an important role in regulating apoptosis and they include the extracellular signal-regulated kinases (ERKs) and the p38 MAPKs (Robinson and Cobb, 1997), which have anti- and pro-apoptotic functions, respectively. This demonstrates that the regulation of follicular atresia is complex and involves the interaction of several signalling pathways.

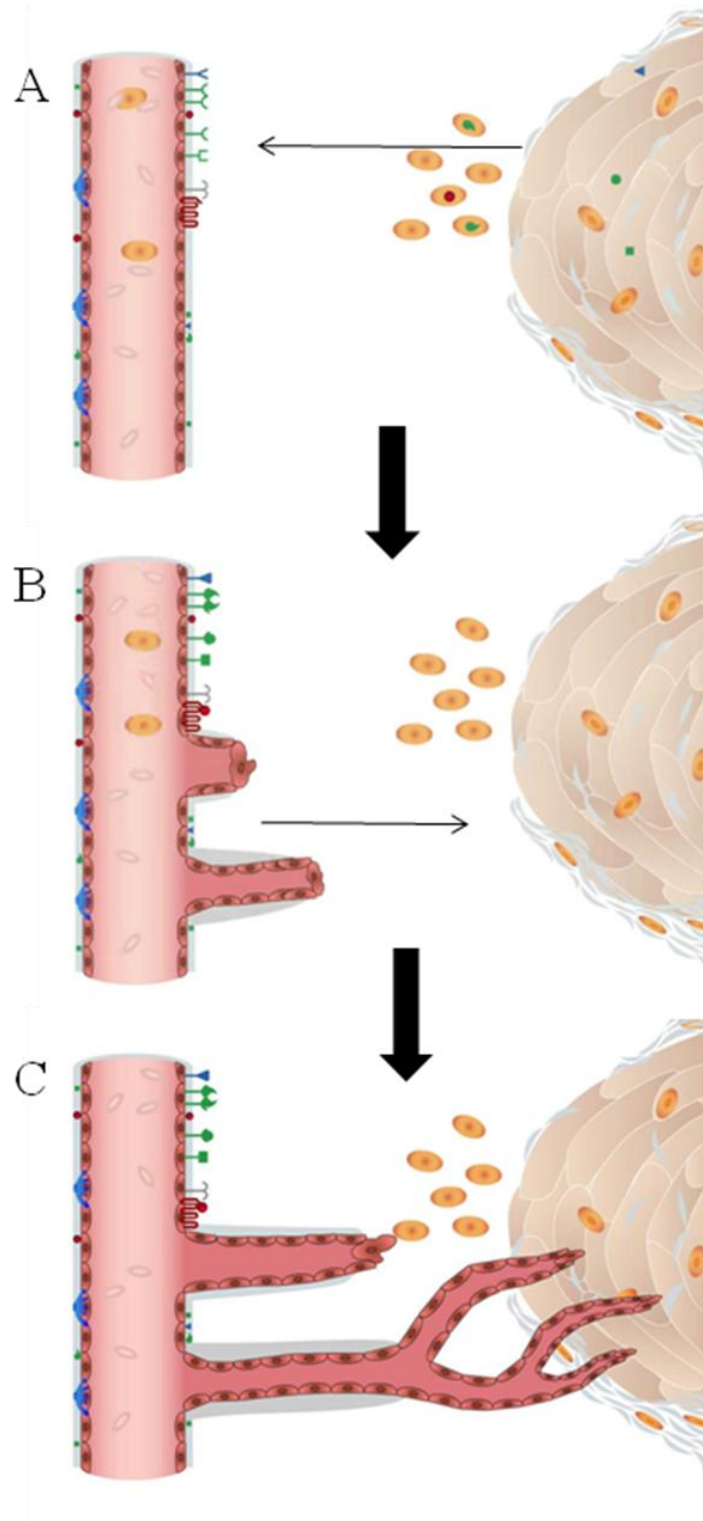
## **1.3 Angiogenesis in the ovary**

There are two main methods of blood vessel development and they are vasculogenesis and angiogenesis. Vasculogenesis is the formation of blood vessels during embryogenesis by endothelial progenitors such as angioblasts. These

endothelial progenitors migrate, sometimes to distant sites, differentiate into endothelial cells and then form the vascular plexus (Risau, 1997, Carmeliet, 2004). Once the vascular plexus has been formed new vasculature develops by the process of angiogenesis, which is the growth of new blood vessels by proliferation and migration of pre-existing blood vessels. Pivotal research into the field of angiogenesis first suggested that angiogenic inhibitors could be used to treat cancer and other diseases dependent on aberrant angiogenesis (Folkman et al., 1971). This crucial research was followed by studies showing that tumours are only able to grow when they are able to attract blood vessels from the existing vasculature surrounding the tumour (Gimbrone et al., 1972).

Angiogenesis is a complex process that requires the breakdown of existing blood vessel walls followed by the migration and proliferation of endothelial cells to form the new vessels. For this to occur the extracellular matrix needs to be degraded and proteinases of the matrix metalloproteinase (MMP) family degrade basement membrane collagens, thus allowing endothelial cells to migrate and proliferate (Bendeck, 2004). These new vessels are comprised of endothelial cells and one or more enveloping layers of supporting cells, usually pericytes or smooth muscle cells (Chantrain et al., 2006). These surrounding pericytes are first found at the advancing tips of endothelial sprouts and once the new vessels are formed these pericytes then extend along the length of the blood vessels and stabilise them (Bergers and Song, 2005, Hall, 2006, Robinson et al., 2009, Raza et al., 2010).

In the healthy adult the endothelial cells of established vessels are quiescent but the migration and proliferation of endothelial cells is required for angiogenesis to occur. The proliferation of endothelial cells is stimulated through the binding of vascular endothelial growth factor (VEGF) to its receptors VEGFR1 and VEGFR2, and occurs in the endothelial cells behind the quiescent tip of the extending blood vessel (Klagsbrun and D'Amore, 1991). The process of angiogenesis is complete when the endothelial cells stop dividing and start to adhere to each other to form new blood vessels. Pericytes are then recruited to surround and stabilise the new blood vessels, allowing blood to begin to flow to and from these vessels.



**Figure 1.3 Diagram of angiogenesis.**

*Follicle release pro-angiogenic factors such as VEGF when they reach the preantral stage of development and they travel to nearby blood vessels and bind to their receptors (A). This stimulates the breakdown of the existing blood vessel wall and the endothelial cells and pericytes from this vessel proliferate and migrate towards the follicle (B), and form new blood vessels in the theca of the developing follicle (C).*

### **1.3.1 Physiological angiogenesis**

Vasculogenesis and angiogenesis are essential processes during embryonic and foetal development when organ development is occurring. However, they are rare in the healthy adult as there is very little physiological vessel growth and remodelling, except during wound healing and in the female reproductive system, where angiogenesis occurs in a cyclical manner.

Angiogenesis is a pivotal event in both bone and organ formation during embryogenesis (Gerber et al., 1999) and VEGF mediated angiogenesis has been shown to be critical for both of these processes. In fact, the inhibition of VEGF can cause abnormal organ development and tissue hypoxia in neonatal life. Although physiological angiogenesis is rare in the healthy adult, the female reproductive system undergoes serial angiogenesis and vessel regression during both follicle development and the establishment and regression of the corpus luteum.

#### ***1.3.1.1 Follicular angiogenesis***

Primordial and primary follicles do not have their own blood supply so angiogenesis is required for follicular growth and development (Robinson et al., 2009). As follicles reach the preantral stage of development endothelial cells are recruited from the ovarian stroma to form vascular sheaths within the thecal layer. These vascular sheaths consist of two concentric networks of blood vessels located in the theca interna and the theca externa (Findlay, 1986, Hunter et al., 2004). The network of arterioles and venules in the theca externa send branches into the theca interna to form the inner capillary plexus. These capillaries are located immediately outside the basement membrane that separates the granulosa and thecal layers and they do not

penetrate the granulosa cell layer until after ovulation has occurred (Findlay, 1986, Reynolds et al., 1992, Gordon et al., 1995, Augustin, 2000, Jaffe, 2000, Fraser, 2006).

This cyclical angiogenesis is tightly regulated by a variety of pro- and anti-angiogenic factors, including VEGF which is considered to be the key factor involved in the stimulation of angiogenesis (Tamanini and De Ambrogi, 2004). VEGF and its receptors have been localized in the ovary, especially in endothelial cells (Phillips et al., 1990, Shweiki et al., 1993, Hunter et al., 2004), and have been shown to be stimulated by FSH and LH (Garrido et al., 1993, Christenson and Stouffer, 1997, Hazzard et al., 1999), suggesting that the expression of VEGF could be hormonally regulated. Additionally, it has been shown that the inhibition of gonadotrophin secretion by a GnRH antagonist results in decreased VEGF expression, further supporting the suggestion that VEGF could be hormonally regulated (Taylor et al., 2004). Various studies have highlighted the role of VEGF in ovarian physiology by demonstrating its essential role in follicular development, as well as its impact upon angiogenesis (Geva and Jaffe, 2000, Danforth et al., 2003, Zimmermann et al., 2003, Fraser and Wulff, 2003, Fraser et al., 2005a, Fraser et al., 2006, Abramovich et al., 2006, Taylor et al., 2007).

### **1.3.2 Pathological angiogenesis**

Although angiogenesis is rare in the healthy adult it is a common feature of many pathological conditions, including coronary artery disease and tumour growth. In addition, in the reproductive system aberrant angiogenesis is involved in



endometriosis, polycystic ovary syndrome (PCOS), ovarian hyperstimulation syndrome (OHSS) and pre-eclampsia (Smith, 1998, Geva and Jaffe, 2000, Folkman, 2001).

Tumours cannot grow larger than 1-2mm<sup>3</sup> in size without developing their own blood supply (Folkman et al., 1971, McDougall et al., 2006) as oxygen and nutrients from the surrounding vasculature cannot penetrate tissues larger than this. Therefore, angiogenesis is a crucial process in tumour growth. Tumours secrete various pro-angiogenic factors, such as VEGF, which stimulate the growth of new blood vessels towards the tumour, allowing them to be supplied with nutrients and gases.

In ovarian conditions that involve aberrant angiogenesis the expression of VEGF is often altered when compared to its physiological regulation. In women with PCOS the expression of VEGF is up-regulated (Agrawal et al., 1998a, Agrawal et al., 1998b) and it has been found to be present in the ascetic fluid from women with OHSS and with ovarian cancer (Gordon et al., 1995), suggesting that VEGF is a key factor involved in stimulating pathological, as well as physiological, angiogenesis.

### **1.3.3 Angiogenic growth factors**

Angiogenesis is a complex process that requires a careful balance between pro- and anti-angiogenic factors. There are many factors involved in the stimulation of angiogenesis but VEGF is considered to be the key factor involved in inducing angiogenesis (Ferrara, 2004). However, the angiopoietins (Ang-1 and Ang-2), bFGF, hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ), tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) and TGF $\beta$

have also been shown to be involved in the positive regulation of angiogenesis (Folkman and Shing, 1992, Yancopoulos et al., 2000).

Hypoxia is an inducer of angiogenesis and it results increased levels of HIF1 $\alpha$  by inhibiting the degradation of HIF1 $\alpha$  and by stimulating the expression of HIF1 $\alpha$  (Boonyaparakob et al., 2005). As the expression of HIF1 $\alpha$  increases, VEGF production is stimulated, resulting in the induction of angiogenesis (Berra et al., 2000, Mukhopadhyay and Datta, 2004, Liao and Johnson, 2007, Fong, 2009). *In vivo* lack of oxygen increases hypoxia which results in the stimulation of HIF1 $\alpha$  and therefore increases angiogenesis. However, several studies have indicated that treatment with anti-angiogenic factors can inhibit the expression and/or activity of HIF1 $\alpha$  (Semenza, 2000, Mabeesh et al., 2003, Ricker et al., 2004, Dunn et al., 2009, Ban et al., 2010, Semenza, 2010). The inhibition of HIF1 $\alpha$  by RNA interference reduces the level of angiogenesis, showing that HIF1 $\alpha$  plays an important role in stimulating angiogenesis (Kim et al., 2010). As HIF1 $\alpha$  plays an important role in promoting angiogenesis it was hypothesised that anti-angiogenic compounds could be inhibiting HIF1 $\alpha$  as a mechanism of angiogenesis inhibition. The use of the *in vitro* system would allow the elucidation of the potential mechanisms of anti-angiogenesis via the interaction with HIF1 $\alpha$ .

#### **1.3.3.1 Vascular endothelial growth factor (VEGF)**

VEGF was originally identified as vascular permeability factor (VPF) due to its ability to stimulate vascular leakage and induce fenestrations in the endothelial cells of capillaries (Senger et al., 1983) however, it was later shown that a factor, named

VEGF, could stimulate angiogenesis (Ferrara and Henzel, 1989, Connolly et al., 1989). The discovery that the DNA sequences encoding both VEGF and VPF were identical (Keck et al., 1989, Leung et al., 1989) lead to the realisation that both VPF and VEGF were the same factor, and it was henceforth referred to as VEGF.

VEGF is part of a family of heparin-binding dimeric glycoproteins that include VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor. The VEGF that was initially discovered was termed VEGF-A and is the member of the family that is commonly referred to as VEGF. VEGF has various isoforms that result from alternative splicing of the VEGF gene and they are VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>165</sub>, VEGF<sub>183</sub>, VEGF<sub>189</sub> and VEGF<sub>206</sub> (Bates and Jones, 2003, Ferrara, 2004, Tammela et al., 2005). VEGF<sub>165</sub> and VEGF<sub>121</sub> are the predominant isoforms in the human ovary (Fujimoto et al., 1998, Otani et al., 1999, Ferrara, 2004). VEGF binds to the receptors VEGFR1 and VEGFR2 and the exons encoding the binding domains for these receptors, exons 3 and 4 respectively, are present in all of the VEGF isoforms (Keyt et al., 1996). In addition, there have been recent reports of additional splicing to produce other VEGF isoforms that have either slightly (VEGF<sub>xxx</sub>) or very (VEGF<sub>xxxb</sub>) different properties to the common VEGF isoforms (Bates and Jones, 2003). Although VEGF plays an important role in stimulating angiogenesis, recent studies have suggested that the VEGF<sub>xxxb</sub> isoforms have anti-angiogenic activity (Glass et al., 2006).

All of the members of the VEGF family bind to cell surface receptors (VEGFRs) which activate an intracellular kinase domain and trigger a cellular response. VEGF

exerts its effects mainly through binding to VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1) although it can also bind to neuropilin-1 and -2 (Ferrara and Keyt, 1997, Bates and Jones, 2003, Ferrara, 2004, Tammela et al., 2005, Pandya et al., 2006).

VEGFR1 and VEGFR2 have seven immunoglobulin (Ig)-like regions in the extracellular domain, a single-transmembrane region and a consensus tyrosine kinase sequence with a kinase-insert domain (Tammela et al., 2005). Although they are structurally similar, VEGFR1 and VEGFR2 have different signalling and biological properties and bind to VEGF with different, but high, affinities (de Vries et al., 1992). VEGFR2 has been shown to bind VEGF with lower affinity than VEGFR1, however, studies have shown that it is the key receptor involved in triggering angiogenesis and vascular permeability (Tammela et al., 2005, Guo et al., 2010). The role of VEGFR1 is less clear but it is thought to mediate cell migration and vascular permeability. In addition there is good evidence that it acts as a decoy receptor to negatively regulate the activity of VEGF by binding to it and sequestering it (Park et al., 1994).

The expression of VEGF mRNA and protein is up-regulated by many cytokines and growth factors including epidermal growth factor (EGF), TGF $\alpha$  and TGF $\beta$ , bFGF, interleukin (IL)-1 and IL-6 and insulin-like growth factor-1 (IGF-1) (Warren et al., 1996, Ferrara and Davis-Smyth, 1997). VEGF expression is also stimulated by the gonadotrophins FSH and LH (Christenson and Stouffer, 1997) and studies using a GnRH antagonist have shown that the blockage of gonadotrophin secretion resulted

in decreased VEGF expression and inhibited angiogenesis in dominant follicles (Taylor et al., 2004). The production of VEGF is also regulated by hypoxia. Hypoxia blocks the degradation of HIF1 $\alpha$  and the increased activity of HIF1 $\alpha$  induces expression of VEGF and therefore increased angiogenesis (Duncan et al., 2008). Stimulation of VEGFR1 by VEGF results in the autophosphorylation of the receptor and promotion of cell migration (Fong et al., 1995). VEGF stimulation of VEGFR2 leads to autophosphorylation of the receptor, activation of the MAP kinase cascade and stimulation of endothelial cell proliferation (Fong et al., 1995).

VEGF plays a crucial role in angiogenesis and studies in knockout mice have shown that the inactivation of VEGF results in embryonic lethality, confirming the essential role of VEGF in the induction of angiogenesis (Carmeliet et al., 1996, Ferrara et al., 1996). Many studies have investigated the effect of inhibiting or neutralising VEGF on angiogenesis and have shown that vessels do not develop without VEGF being expressed. Furthermore, studies in the ovary have shown that when VEGF is inhibited follicles do not undergo follicle development and ovulation is blocked, showing the vital role VEGF has in both ovarian angiogenesis and ovarian function (Wulff et al., 2001a, Wulff et al., 2001b, Fraser et al., 2005a, Fraser et al., 2005b, Fraser et al., 2006, Taylor et al., 2007). In addition, the observation that VEGF is up-regulated in many pathological conditions (Krasnow et al., 1996, Agrawal et al., 1998b) has led to the proposed use of anti-VEGF treatments, such as Bevacizumab and Aflibercept (VEGF Trap), to inhibit pathological angiogenesis (Strawn et al., 1996, Presta et al., 1997, Taylor et al., 2007).

**1.3.3.2 Angiopoietins**

The angiopoietins are considered to be the most important class of proteins that act in concert with VEGF to produce and maintain stable blood vessels (Maisonpierre et al., 1997). The angiopoietins are cytokine glycoproteins that act in a competitive manner via the tyrosine kinase receptor Tie-2 (Maisonpierre et al., 1997). There are four known angiopoietins; Ang-1, Ang-2, Ang-3 and Ang-4; with the best characterised being Ang-1 and Ang-2. All four bind to the receptor Tie-2 however, Tie-1 is an orphan tyrosine kinase receptor that does not appear to bind to any of the angiopoietins (Partanen et al., 1992, Kontos et al., 2002).

Ang-1 and Tie-2 are widely expressed, including in the ovarian follicle of the rodent, bovine and primate, however expression of Ang-1 and its receptor varies from species to species (Maisonpierre et al., 1997, Wulff et al., 2001a, Hayashi et al., 2003, Abramovich et al., 2009). In the marmoset *in situ* hybridisation has shown that Tie-2 is expressed in the vasculature of the thecal layer of antral follicles but is also expressed in granulosa cells of primordial, primary and preantral follicles (Wulff et al., 2001a). Ang-1 and Tie-2 mRNA has been shown by RT-PCR to be expressed in the thecal layer of bovine follicles but granulosa cells express mRNA for Ang-1 only (Hayashi et al., 2003). Recently it has been shown by immunohistochemistry and western blotting that Ang-1 and Tie-2 expression is confined to the endothelial cells in the thecal layer of rat follicles and is not present in granulosa cells at any stage of follicular development (Abramovich et al., 2009). Overall, it is generally thought that the expression of Tie-2 is confined to endothelial cells. Ang-1 is primarily involved in supporting and stabilising newly developed blood vessels (Thurston, 2002). In

addition, Koblizek *et al* showed that Ang-1 can stimulate capillary sprouting and that after treatment with soluble Tie-2 receptor domains, this sprouting was inhibited (Koblizek et al., 1998), supporting the evidence for Ang-1 regulating angiogenesis through interaction with Tie-2. The pattern of expression of both Ang-1 and Tie-2 in the ovary suggests that Ang-1/Tie-2 signalling may play a role in regulating the cyclical angiogenesis that occurs in the female reproductive system.

Ang-2 has considerable sequence homology to Ang-1 and the balance between the expression of Ang-1 and Ang-2 during angiogenesis is important. The expression of Ang-2 has been localised to the ovary, placenta and uterus (Maisonpierre et al., 1997). Studies have shown that the over-expression of Ang-2 can disrupt blood vessel formation in the embryo, much like in Tie-2 and Ang-1 knockout mice, indicating that Ang-2 also plays an important role in the regulation of angiogenesis. Ang-2 can both aid the development of new blood vessels and aid vascular regression, dependent on the circumstances, through antagonism of the Tie-2 receptor (Maisonpierre et al., 1997, Asahara et al., 1998, Witzenbichler et al., 1998). Ang-2, when acting with VEGF, can stimulate angiogenesis by interfering with endothelial cell-pericyte interactions and aiding the degradation of the extracellular matrix, thus allowing the endothelial cells to migrate from the existing blood vessels. In the absence of VEGF, Ang-2 inhibits angiogenesis, induces vessel regression and stimulates endothelial cell death (Maisonpierre et al., 1997). Ang-2 also reduces the effect of Ang-1 by competing for receptor binding. Little is known about the actions of Ang-3 and Ang-4 but they can both positively and negatively influence angiogenesis, dependent on the conditions (Lee et al., 2004, Kim et al., 2007).

### **1.3.4 Anti-angiogenic factors**

Angiogenesis is regulated by a careful balance of pro- and anti-angiogenic factors (Ferrara, 2004, Tamanini and De Ambrogi, 2004, Greenaway et al., 2007). Endogenous angiogenesis inhibitors play an important role in regulating both physiological angiogenesis and pathological angiogenesis and many angiogenesis inhibitors have been described. They include interferons, platelet factor 4,  $\text{TNF}\alpha$ , thrombospondin-1,  $\text{TGF}\beta$  and angiostatic steroids (Klagsbrun and D'Amore, 1991). Endogenous angiogenesis inhibitors, such as angiostatin, arresten and endostatin, are often generated from larger molecules, that are not themselves inhibitors of angiogenesis, and they are released from these larger molecules to maintain normal blood vessel development (Ribatti, 2009).

Synthetic compounds have been developed that have anti-angiogenic activity and they have potential use as therapeutics in pathological angiogenesis. Exogenous angiogenesis inhibitors have been identified through the use of cell culture assays (Ingber et al., 1990) and there are now numerous synthetic compounds available to inhibit angiogenesis, including antibodies to VEGF (Kim et al., 1993, Fraser et al., 2000, Dickson et al., 2001) and inhibitors of VEGF such as Aflibercept (Wulff et al., 2001a, Wulff et al., 2001b, Wulff et al., 2002, Fraser et al., 2006). The development of angiogenesis inhibitors is an area of great clinical interest as angiogenesis plays an important role in many pathological conditions.

#### **1.3.4.1 Thrombospondin-1 (TSP-1)**

The members of the thrombospondin family are large extracellular matrix glycoproteins that are secreted by many cell types and can act as autocrine factors.



There are five members of the TSP family and they are TSP-1 and TSP-2 which are trimers, and TSP-3, TSP-4 and TSP-5 which are pentamers (Bonney et al., 2008). The members of the TSP family have a high degree of homology but are differentially expressed and have different promoters, suggesting that they are likely to have different functions. TSP-1 and TSP-2 are the best described members of the TSP family and TSP-1 has been widely studied since its discovery.

TSP-1 can bind to a variety of receptors and therefore has a wide range of effects due to the different signalling pathways that it activates (Lawler, 2002). Although TSP-1 activates a variety of receptors its main function is as an endogenous angiogenesis inhibitor with anti-cell migration properties (Tamanini and De Ambrogio, 2004, Ferrara, 2004, Greenaway et al., 2007). The anti-angiogenic activity of TSP-1 has been isolated to a smaller sequence within TSP-1 known as thrombospondin type 1 repeats (TSRs) (Zhang and Lawler, 2007). Three TSRs are found in TSP-1 and TSP-2 but they are not found in TSP-3, TSP-4 and TSP-5. The regulation of the anti-angiogenic effect of TSP-1 is complex and both direct and indirect effects on endothelial cells are involved. TSP-1 acts indirectly on inflammatory cells and myofibroblasts and also directly inhibits the migration of endothelial cells and induces their apoptosis (Lawler, 2002). TSP-1 inhibits angiogenesis both *in vitro* and *in vivo* and studies have shown that treatment with TSP-1 renders endothelial cells unable to respond to many pro-angiogenic factors (Lawler, 2002, Folkman, 2004). Several studies have used knock-out mice to show that the absence of TSP-1 leads to increased vascularisation (Lawler et al., 1998, Lawler et al., 2001, Wang et al., 2003,

Sund et al., 2005, Greenaway et al., 2007), providing the first evidence for a role of TSP-1 *in vivo* as an inhibitor of angiogenesis.

The anti-angiogenic effects of TSP-1 are mediated through binding to the cell surface receptors CD36 and CD47 (integrin-associated protein) (Dawson et al., 1997, Lawler, 2002). CD36 is expressed on many cell types including endothelial cells and platelets. CD36 interacts with TSP-1 but can also bind low-density lipoproteins and collagens I and V (Asch et al., 1987, Tandon et al., 1989, Kehrel et al., 1993). During platelet activation CD36 is dephosphorylated which blocks collagen binding and allows TSP-1 to bind to the receptor (Bonney et al., 2008). In addition, studies with monoclonal antibodies against CD36 demonstrated that the expression of TSP-1 on platelets is also inhibited. Studies in CD36 deficient mice have confirmed the importance of this receptor in TSP-1 signalling but it is not the sole receptor for TSP-1 (Lawler, 2002, Bonney et al., 2008). Integrin-associated protein (IAP) or CD47 is present on the surface of many cell types and interacts with integrins and heterotrimeric G proteins (Gao et al., 1996, Chung et al., 1997, Wang and Frazier, 1998, Frazier et al., 1999). CD47 is a member of the immunoglobulin receptor superfamily and it has been shown that TSP-1 signalling via CD47 inhibits nitric oxide (NO) signalling (Isenberg et al., 2009). As NO signalling promotes endothelial cell health and inhibits the activation of inflammatory cells, signalling via CD47 could be involved in the stimulation of endothelial cell apoptosis by TSP-1. This is supported by evidence that animals without TSP-1 or CD47 show enhanced tissue survival in hypoxic conditions (Isenberg et al., 2009).

In the ovary, TSP-1 is most highly expressed during the preantral and early antral stages of follicular development (Petrik et al., 2002, Greenaway et al., 2005), and studies in rat, bovine and marmoset models have demonstrated a decrease in both TSP-1 and CD36 expression as follicular development progresses (Petrik et al., 2002, Greenaway et al., 2005, Thomas et al., 2008), leading to the suggestion that TSP-1 acts to limit any overgrowth of the vasculature that develops in response to the high levels of pro-angiogenic factors expressed at these stages. In addition, TSP-1 is often produced at a rate that is inversely proportional to the production of VEGF and it has been shown to bind to VEGF and inhibit its action (Greenaway et al., 2007), suggesting that the anti-angiogenic action of TSP-1 could, at least in part, be regulated through interaction with VEGF.

A previous descriptive study has made the interesting observation that TSP-1 mRNA and protein is up-regulated during follicular atresia *in vivo* (Thomas et al., 2008), suggesting that TSP-1 may be involved in the cessation of angiogenesis in follicles undergoing atresia. Alternatively, TSP-1 may be an autocrine/paracrine factor acting on granulosa and/or endothelial cells to promote follicular atresia, as the CD36 receptor for TSP-1 is present in both cell types (Thomas et al., 2008).

#### **1.3.4.2 2-methoxyestradiol (2-ME)**

2-methoxyestradiol (2-ME) is a naturally occurring derivative of 17 $\beta$ -estradiol (Shang et al., 2001) and it has been shown to have anti-tumour and anti-angiogenic activity (Fotsis et al., 1994, Pribluda et al., 2000, Ricker et al., 2004). It is synthesised by hydroxylation of estradiol at the 2-position and the subsequent O-

methylation by catechol-o-methyltransferase (Pribluda et al., 2000, Dahut et al., 2006). Although 2-ME is an oestrogen derivative it has low binding affinity for oestrogen receptors  $\alpha$  and  $\beta$ , and its anti-proliferative action is not mediated through an oestrogen-mediated pathway (LaVallee et al., 2002).

As the ovary produces high levels of oestrogen it is likely that 2-ME is produced in the ovary. A previous study has shown that 2-ME is present in porcine follicular fluid, with concentration increasing as folliculogenesis progresses (Basini et al., 2007). As 2-ME is present in ovarian follicles it is likely that 2-ME plays a role in regulating follicular angiogenesis. In addition, several studies have shown that 2-ME can induce apoptosis (Pribluda et al., 2000) suggesting that the anti-proliferative activity of 2-ME is likely to be due to the initiation of apoptosis and that 2-ME could have an extravascular role in the ovary. 2-ME has been demonstrated to directly inhibit the proliferation of endothelial cells and tumour cells and can indirectly reduce angiogenesis through the inhibition of HIF1 $\alpha$  protein expression, as well as inhibiting its translocation to the nucleus (Mabjeesh et al., 2003, Ricker et al., 2004, Lu et al., 2010). As 2-ME has previously been shown to inhibit the activity of HIF1 $\alpha$  it is possible that the anti-proliferative action of 2-ME could be due to negative regulation of the cytoprotective activity of VEGF via the inhibition of HIF1 $\alpha$ . 2-ME has also been shown to activate the caspase cascade (Mooberry, 2003a, Mooberry, 2003b), induce p38 activation (LaVallee et al., 2003, Shimada et al., 2003) and inhibit Akt (Lin et al., 2007). Therefore it is possible that 2-ME negatively regulates proliferation due to the stimulation of pro-apoptotic mediators and/or the suppression

of pro-survival pathways, but the mechanisms of these extravascular effects remain unclear.

### **1.3.5 Extravascular action of angiogenic factors**

This chapter has discussed the roles of various pro- and anti-angiogenic factors in regulating angiogenesis. However, some of these factors have been suggested to have extravascular roles within the ovary, including VEGF, TSP-1 and Ang-1 (Jimenez et al., 2000, Greenaway et al., 2004, Greenaway et al., 2007, Parborell et al., 2008). Many of the factors and their receptors are expressed on granulosa cells, as well as on endothelial cells, and can stimulate non-angiogenic pathways, but the mechanisms of these extravascular effects remain unclear.

In addition to its pro-angiogenic effect, VEGF has been shown to have a cytoprotective effect in the bovine ovary. VEGF interacts with VEGFR2 to reduce apoptosis and to stimulate the expression of pro-survival mediators in endothelial and granulosa cells (Gerber et al., 1999, Greenaway et al., 2004). Moreover, treatment with inhibitors of VEGF, such as Aflibercept, results in an increase in follicular atresia suggesting that VEGF plays an important extravascular role in follicular development (Greenaway et al., 2004, Abramovich et al., 2006). It is possible that the inhibition of angiogenesis alone would be sufficient to induce follicular atresia as the reduced blood supply would prevent delivery of hormones, nutrients and gases, resulting in increased apoptosis. However, it is thought that VEGF exerts its cytoprotective effect by directly inhibiting apoptosis in granulosa cells as it blocks the release of CAD from the CAD:ICAD complex which, when released, causes

fragmentation of DNA (Kosaka et al., 2007). Furthermore, it has been shown that the inhibition of VEGF can inhibit granulosa cell proliferation (Doyle et al., 2010) and migration, suggesting that VEGF could play an important role in regulating granulosa cell function and/or survival.

Treatment of rat ovaries *in vivo* with an antibody to Ang-1 results in the promotion of follicular atresia (Parborell et al., 2008), suggesting that Ang-1 could have a role in cell survival. The expression pattern of Ang-1 and Tie-2 suggests that they could be regulating cell survival via autocrine/paracrine signalling. The anti-apoptotic activity of Ang-1 is thought to be mediated by the phosphatidylinositol 3'-kinase (PI3K)/Akt signalling pathway, downstream of the Tie-2 receptor (Augustin et al., 2009). This signalling pathway is a key mediator of cell survival so stimulation of this pathway would induce a pro-survival effect. Furthermore, Kim et al. (Kim et al., 2000) demonstrated that Ang-1 induces PI3K-dependent phosphorylation of Akt, resulting in decreased apoptosis in serum deprived human umbilical vein endothelial cells (HUVECs). Previous studies have investigated the effects of both Ang-1 and Ang-2 on ovarian function *in vivo* and have shown that the addition of Ang-1 has little detrimental effect on ovarian function but that treatment with Ang-2 resulted in deterioration of the mature, preovulatory follicle(s) and inhibited ovulation (Xu and Stouffer, 2005). Additionally, in the rat ovary the expression of Ang-2 mRNA is increased in follicles undergoing atresia (Maisonpierre et al., 1997). These results suggest that Ang-2 could have a role in regulating follicle survival and that Ang/Tie signalling is important for promoting follicle health.

Since CD36 is expressed in granulosa cells, it is possible that TSP-1 may be acting directly on these cells in an autocrine fashion to promote follicular atresia via an apoptotic mechanism (Thomas et al., 2008). Induction of apoptosis by TSP-1 requires the sequential activation of the CD36 receptor, p59fyn, caspase-like proteases and p38 mitogen-activated protein kinases (MAPK) (Jimenez et al., 2000). It has been suggested that TSP-1 could have an extravascular role in regulating follicular apoptosis (Jimenez et al., 2000, Nor et al., 2000, Greenaway et al., 2007). Additionally studies have suggested that TSP-1 can interact with the survival mediators ERK and MAPK (Wilson et al., 1999, Sengupta et al., 2004, Tan et al., 2009), which supports the suggestion that TSP-1 could play a role in follicle survival. Using a granulosa cell line, Greenaway and colleagues showed that TSP-1 treatment decreased VEGF levels and rendered cells more susceptible to TNF- $\alpha$ -induced apoptosis (Greenaway et al., 2007). TSP-1 acts mainly via the receptor CD36 and since CD36 receptors are present on granulosa cells, it is likely that the promotion of follicular apoptosis by TSP-1 is due to a direct effect on the granulosa cells.

Treatment with 2-ME increases the stimulation of activated caspase-3, one of the main molecules involved in the induction of apoptosis, so it is likely that 2-ME also regulates the expression of other pro- or anti-apoptotic mediators. It was anticipated that 2-ME could have an effect on the expression of pERK and/or p38 (LaVallee et al., 2003, Mooberry, 2003a, Mooberry, 2003b, Shimada et al., 2003, Lin et al., 2007) as they are key factors involved in anti- and pro-apoptotic pathways.

Although the main role of these factors is in regulating angiogenesis, they have been shown to act on non-vascular cells and have extravascular actions within the ovary. Therefore the role of angiogenic factors in normal ovarian function will be investigated in this thesis. As many of the factors either stimulate or inhibit pro-survival mediators, the expression of pERK, p38 and FOXO1 will be investigated following treatment with the different angiogenic factors. Akt signalling leads to inactivation of the forkhead transcription factor, FOXO1 which blocks cell division and promotes apoptosis (Augustin et al., 2009) and has been shown to be expressed on granulosa cells of growing follicles (Bastie et al., 2005). The expression of FOXO1 is regulated by the gonadotrophins; FSH and estradiol increases the expression of FOXO1, whereas LH decreases its expression (Liu et al., 2009). FOXO1 is also involved in regulating FSH-mediated granulosa cell proliferation and differentiation, suggesting that FOXO1 may play a critical role in granulosa cell function. Therefore, alteration in the expression of these molecules could indicate an extravascular role for the angiogenic factors in follicle survival and development.

### **1.3.6 Manipulation of angiogenesis**

There are many models available to investigate angiogenesis, including both *in vitro* and *in vivo* systems. *In vitro* angiogenesis assays allow the analysis of the isolated processes of angiogenesis to be carried out, enable the identification of the direct effect of compounds on endothelial cell function, are more easily quantified and are generally less expensive to perform than *in vivo* assays. However, *in vivo* assays are more representative of physiological angiogenesis and give a whole system view of the effect of compounds on angiogenesis. Therefore, both *in vitro* and *in vivo* assays



are important for angiogenesis research despite their various advantages and disadvantages.

#### **1.3.6.1 *In vitro* assays**

*In vitro* angiogenesis assays have several critical advantages over *in vivo* angiogenesis assays and they are frequently used in research due to their ease of manipulation and quantification. There are several *in vitro* systems commonly used and they include assays designed to investigate one specific process in angiogenesis and also those designed to investigate the angiogenic process as a whole.

Angiogenesis begins with the degradation of the extracellular matrix and this involves the action of proteases such as the MMPs (Davis and Senger, 2005). To study the process of matrix degradation, matrix invasion or zymogen assays are used. This technique is relatively inexpensive and provides information about MMP activity. However, it is time consuming and is not suitable for large-scale screening.

After basement membrane degradation occurs endothelial cells migrate from the existing blood vessels into the surrounding tissue. This process is investigated using a variety of assays, including scratch wound assays, transwell or Boyden assays and migration assays (Staton et al., 2004, Goodwin, 2007). These assays are designed to allow quantification of the rate at which endothelial cells migrate into an area lacking endothelial cells. Whilst these assays have advantages they also have limitations. Scratch wound assays allow multiple quantifications to be carried out at different time points and can easily be adapted for large-scale screening, however, they can be

difficult to quantify and are highly variable (Auerbach et al., 2003, Goodwin, 2007).

Migration assays are inexpensive to perform and allow the differentiation between chemokinesis and directed cell migration but are variable and difficult to quantify. Transwell assays are the easiest of the migration assays to quantify and are sensitive and highly reproducible, but the membranes used in this assay are expensive (Goodwin, 2007). There are also assays available for the investigation of endothelial cell proliferation and survival but they tend to be used in conjunction with other angiogenesis assays.

Despite the availability of assays for the individual processes that make up angiogenesis, the main *in vitro* angiogenesis assays currently used to investigate angiogenesis, excepting human umbilical vein endothelial cell (HUVEC) assays (Nehls and Drenckhahn, 1995a, Nehls and Drenckhahn, 1995b, Nakatsu and Hughes, 2008), tend to be organ culture assays including the rat aortic ring assay, the chick aortic arch assay and the mouse metatarsal assay (Auerbach et al., 2000, Auerbach et al., 2003, Staton et al., 2004, Goodwin, 2007), and whilst all of these methods have enhanced the understanding of angiogenesis, and of the mechanisms involved in it, they also have limitations.

Angiogenesis is primarily microvascular event so the use of large vessels, such as in the rat aortic ring assay, the chick aortic arch assay and HUVEC assays is not truly representative of *in vivo* angiogenesis. The use of tissues that have been obtained from growing embryos is also not representative of physiological angiogenesis as the cells are undergoing rapid cell division and proliferation, before explantation.

Therefore the vessel outgrowth seen is not representative of the stimulation of angiogenesis from non-proliferative endothelial cells (Staton et al., 2004, Goodwin, 2007). In addition, these assays are time consuming – often taking up to 2 weeks to provide results (Auerbach et al., 2000, Auerbach et al., 2003, Staton et al., 2004, Goodwin, 2007) and many of these assays are limited by variability of the tissues used, making it necessary to use large numbers to achieve significance. HUVEC assays (Kubota and Tamauchi, 1988, Donovan et al., 2001, Auerbach et al., 2003) have proven useful in defining the mechanisms involved in the individual stages of angiogenesis, as they are ideal for focussing on one stage of angiogenesis. Moreover, they are much more rapid than other *in vitro* assays and are easily reproducible. However, HUVEC assays share many of the limitations of the other *in vitro* assays and are not suited to the study of the complete angiogenic process.

#### **1.3.6.2 *In vivo* assays**

There are several *in vivo* angiogenesis models used in current research and whilst they are more representative of the stimulation of angiogenesis under normal *in vivo* conditions, they are limited by numerous challenges due to the complexity of the system used and the need to reduce the use of live animals in scientific research. The most commonly used *in vivo* models are the Matrigel plug assay, the corneal assay, the chick chorio-allantoic membrane (CAM) assay and chamber assays including the rabbit ear chamber, dorsal skinfold chamber and cranial window chamber (Auerbach et al., 2000, Akhtar et al., 2002, Auerbach et al., 2003, Staton et al., 2004).

In the Matrigel plug assay, Matrigel (or other gels such as collagen or fibrin) which is liquid at 4°C, allows the injection of cells or test compounds into test animals, where it then solidifies into a plug as Matrigel is solid at body temperature (Auerbach et al., 2000, Akhtar et al., 2002, Staton et al., 2004). Blood vessels then grow into the Matrigel plug, allowing the quantification of angiogenesis as all the vessels present in the plug are newly formed (Auerbach et al., 2003). This assay is easy to perform but has high levels of variability due to the difficulties in forming identical plugs and is time consuming to quantify. The corneal assay was originally developed for use in the rabbit but is now used in a variety of species including rats, guinea pigs and mice (Auerbach et al., 2000, Auerbach et al., 2003). This assay is reliable and can be monitored by direct observation however; the surgery is technically demanding and expensive to perform. The CAM assay is easy to carry out but uses an embryonic model so is not representative of physiological angiogenesis (Klagsbrun and D'Amore, 1991, Auerbach et al., 2000, Auerbach et al., 2003, Staton et al., 2004). Chamber assays enable the determination of blood vessel growth over an extended time period which reduces the number of animals used and are considered a 'gold standard' angiogenesis assay but they are all highly invasive and technically difficult to perform (Auerbach et al., 2000). They are also expensive to carry out and can have reduced visualisation due to the thickness of the skin (Staton et al., 2004).

There are also ovarian models available for the *in vivo* investigation of angiogenesis and the marmoset is a well established model that has been investigated in great detail (Fraser et al., 2000, Fraser et al., 2006, Fraser and Duncan, 2009). The

marmoset has advantages over other non-human primate models (Fraser et al., 1999) as they have high fecundity which allows sufficient animals to be bred in captivity without the need for wild-caught animals, and they are easy to handle and reach sexual maturity quickly (Torii et al., 1996), making them a useful model for reproductive research. They are small in size so only small amounts of compounds are needed to carry out investigations in this species and the ovarian cycle can be easily manipulated. The effects of test compounds on ovarian angiogenesis can be investigated by employing the well established method of using immunohistochemical markers such as bromodeoxyuridine (BrdU) and CD31, to enable the identification of actively proliferating endothelial cells (Wulff et al., 2001a, Wulff et al., 2001b, Fraser et al., 2006, Taylor et al., 2007). This method has been thoroughly investigated and analysed, and it allows the effect on angiogenesis and cellular proliferation to be easily determined. Effects on angiogenesis can also be determined through hormone assays (Smith et al., 1990) and through colour Doppler ultrasonography (Fraser, 2006, Einspanier et al., 2006), allowing results to be determined non-invasively as well as through more traditional methods. The marmoset model has many advantages over other angiogenesis assays as it utilises and manipulates the natural angiogenic properties of the ovary and the angiogenesis that occurs is both natural and physiological.

Overall, the assays currently available for angiogenesis each have their own limitations and the development of a novel *in vitro* angiogenesis assay, that would be representative of *in vivo* angiogenesis, that would be easily reproducible and that would provide a method for mechanistic studies would be a valuable tool in

angiogenesis research. The ovarian follicle is potentially an excellent tissue to utilise for the development of an angiogenesis assay as the tissue is already primed to undergo angiogenesis and the endothelial cells in the thecal layer are ready to sprout. In addition, the angiogenesis in follicles is easy to measure *in vivo* allowing the same system to be interrogated *in vivo* as well as *in vitro*.

#### **1.3.6.3 Development of anti-angiogenic agents**

The development of anti-angiogenic agents has become a focus of cancer therapy and several compounds have been developed (Kerbel and Folkman, 2002). These compounds have a variety of actions including inhibiting the action of pro-angiogenic factors and inhibiting endothelial cell proliferation, migration and survival (Hagedorn and Bikfalvi, 2000, Kerbel, 2001).

Some of these compounds have shown promise in the therapeutic inhibition of angiogenesis, such as antibodies to VEGF and VEGFR2, whilst others have proved disappointing (O'Reilly, 1997, O'Reilly et al., 1997, Coussens et al., 2002, Marshall, 2002). Ferrara and colleagues demonstrated that antibodies to VEGF slowed tumour growth in the mouse model (Kim et al., 1993), and a variety of therapeutic strategies aimed at blocking VEGF or its receptor signalling are currently being developed for the treatment of neoplastic diseases (Alvarez et al., 2010, Delli Carpini et al., 2010, Korpany et al., 2010, Tol and Punt, 2010). VEGF/VEGFR blockade by monoclonal antibodies and inhibition of receptor signalling by tyrosine kinase inhibitors are, to date, the most studied approaches. Bevacizumab (rhuMAb VEGF, Avastin<sup>TM</sup>, Genentech Inc, South San Francisco, CA, USA), a humanised monoclonal antibody

directed at VEGF, is the most advanced in clinical development (Ferrara, 2004). Bevacizumab was approved for use in metastatic cancer by the US Food and Drug Administration in 2004 (Huang et al., 2009) and has now been approved for use in a variety of cancers (Mancuso and Sternberg, 2006, Bossung and Harbeck, 2010, Chamberlain, 2010, Summers et al., 2010). In addition, Aflibercept (Regeneron Pharmaceuticals), a recombinant chimeric protein comprising portions of the extracellular domains of the human VEGFR1 and 2 expressed in sequence with the Fc portion of human Ig (Holash et al., 2002), is currently in clinical trials and has shown promising results (Dixon et al., 2009, Tew et al., 2009, Twardowski et al., 2010).

A correlation between down-regulation of TSP-1 and tumour angiogenesis and invasiveness (Rodriguez-Manzaneque et al., 2001, Miyanaga et al., 2002, Tanaka et al., 2002, Ren et al., 2006), has led to the proposed use of TSP-1 as a therapeutic inhibitor of angiogenesis (Weinstat-Saslow and Steeg, 1994, Streit et al., 1999, Greenaway et al., 2009). The anti-angiogenic activity of TSP-1 has been mimicked by many synthetic compounds and they are usually designed to mimic the activity of the TSR region. A much smaller heptapeptide sequence within TSR-1, GVITRIR, has been mimicked by the modified nonapeptide, ABT-510: Ac-Sar-GV-DalloIle-T-Nva-IRP-ethylamide, which has been shown to be active anti-angiogenically in slowing tumour growth in preclinical models (Anderson et al., 2007, Yang et al., 2007) and shown to reduce VEGF production in a model of ovarian cancer (Greenaway et al., 2009). It has also been demonstrated to be efficacious in the treatment of spontaneously occurring cancer in companion dogs (Rusk et al., 2006),

and has been in clinical trials, mainly as monotherapy (Hoekstra et al., 2005, Gietema et al., 2006, Hoekstra et al., 2006, Markovic et al., 2007).

However, the potency of ABT-510 in human clinical trials was found to be insufficient so a second generation mimetic of the anti-angiogenic activity of TSP-1 has been developed. The thrombospondin mimetic peptide-A-428898 (ABT-898) (Abbott Laboratories, N. Chicago, IL, USA) is a substituted octapeptide, Ac-GV-DalloIle-SQIRP-ethylamide, with greatly increased potency over that of ABT-510 as well as slower clearance in primates. ABT-898 is thus expected to have greater efficacy than the other available TSP-1-mimetic peptides. Overall, much work is being carried out in developing angiogenesis inhibitors as future therapeutics and the development of potent therapeutics with few side effects would have great clinical benefit.

## **1.4 Clinical Relevance of follicular angiogenesis research**

The physiological angiogenesis that occurs in the female reproductive tract is highly organised and is tightly regulated by a balance of pro- and anti-angiogenic factors. However, pathological angiogenesis results from an imbalance of these factors and this can result in severe clinical conditions. Understanding the mechanisms involved in regulating physiological angiogenesis will highlight the importance of each factor involved in this process and could lead to the development of new therapeutics. Novel treatments that can target aberrant ovarian angiogenesis would have a wide range of clinical applications, in conditions such as PCOS, and could benefit many people. Moreover, the elucidation of the role of endogenous and exogenous



angiogenesis inhibitors could indicate lead compounds for further investigation and clinical trials.

In addition, utilising the natural angiogenic properties of the ovarian follicle allows investigation into the role of angiogenic factors in non-vascular cells and could enable the elucidation of their potential extravascular effects within the ovary. The promotion of follicle and oocyte health is a crucial for healthy follicle development and the discovery of novel factors that could promote follicle health may help to improve culture systems for follicular development. This could result in improvements to *in vitro* maturation (IVM) techniques, facilitating the development of novel treatments for infertility.

## 1.5 Aims of the thesis

The aims of this thesis were:

- To develop a novel *in vitro* angiogenesis assay that allows the quantification and manipulation of follicular angiogenesis.
- To elucidate the role of angiogenic factors on follicular angiogenesis.
- To use an *in vitro* angiogenesis assay to investigate the effects of the angiogenic factors on the health of ovarian follicles to determine potential roles for these factors in ovarian function.

- To translate factors of interest from *in vitro* studies to an *in vivo* marmoset monkey model.

This thesis hypothesised that factors involved in regulating angiogenesis may have extravascular roles within the ovary and that an *in vitro* assay utilizing the angiogenic properties of the ovarian follicle would allow this to be investigated.

## **Chapter 2 Materials and Methods**

## **2 Chapter 2 Materials and Methods**

### **2.1 Materials and suppliers**

Unless otherwise stated all reagents were supplied by Sigma-Aldrich Company Ltd (Poole, Dorset, UK).

### **2.2 Experimental models**

Two main experimental models were used for the investigation of follicular angiogenesis, an *in vitro* rodent model and an *in vivo* marmoset model. There are several animal species used in scientific research but rats and mice are the most commonly used. Rodents are easy to handle and have a short ovulatory cycle and gestation, which allows many litters to be produced in a short time period. In addition, they are multi-ovulatory species and as such have high numbers of follicles at all stages of development in the pre-pubertal ovary, making them ideal for the study of follicular angiogenesis. These high numbers of different classes of follicle allow experiments with high n numbers to be carried out whilst minimising the number of animals used.

There are several non-human primate models available for scientific research and although none of them are ideal for research relating to the human, the marmoset has some advantages over other non-human primate models (Fraser et al., 1999). Marmosets have high fecundity, are easy to handle and reach sexual maturity quickly (Torii et al., 1996) making them a useful model for reproductive research. In

addition, the ability to synchronise the ovarian cycle through the induction of luteolysis after injection of prostaglandin enables the study of precise stages in the ovarian cycle (Summers et al., 1985, Duncan et al., 1998, Gilchrist et al., 2001).

### **2.2.1 The rodent ovulatory cycle**

The rat (*Rattus norvegicus*) was the animal model used for the follicle culture and granulosa cell experiments during the course of this thesis. In rats the ovarian cycle starts immediately after the opening of the vaginal orifice. Rats can initially have some irregular cycles (Goldman et al., 2007) but will then settle into a regular ovarian cycle lasting approximately 4-5 days, typically with several ovulations per cycle (Conn MP and S, 1997).

### **2.2.2 The marmoset ovulatory cycle**

The marmoset monkey (*Callithrix jacchus*) was the animal model used for the *in vivo* investigations of follicular angiogenesis. In marmosets the ovarian cycle starts at approximately 14 to 18 months of age (McAnulty, 1994). The ovarian cycle lasts for approximately 28 days, typically with two or three ovulations per cycle (Harding et al., 1982, Hearn, 1983). The follicular phase lasts for approximately 9 to 10 days and it has been demonstrated that it takes 10.7 days on average for the growth of follicles to reach the ovulatory stage after the sudden termination of the corpus luteum with administration of a PGF<sub>2α</sub> analogue (Summers et al., 1985). Marmosets have a luteal phase of approximately 20 days (Harding et al., 1982). In most cycles, 2 or 3 dominant follicles grow at similar rates with ovulation occurring at up to 12-hour intervals (Torii et al., 1996).

## **2.3 Rodent Tissue**

### **2.3.1 Rodent husbandry**

All rodents were housed in the Animal House, Chancellors Building, Edinburgh. Staff at the centre were responsible for the care and maintenance of the animals. Animals were housed under temperature controlled conditions on a 12h light/12h darkness cycle and fed rodent chow *ad libitum*. Handling and treatment of animals were according to the Animals (Scientific Procedures) Act, 1986.

### **2.3.2 Collection of tissue**

Animals were killed by asphyxiation with CO<sub>2</sub> and their ovaries were removed immediately using manual dissection. Follicles were then manually isolated from the ovaries under a laminar flow hood.

### **2.3.3 Treatment regimes**

Administration of treatments was undertaken after isolation of primary tissues. The treatment was added to the medium and tissues were cultured in this medium or in control medium. Specific details of the dose, timing and duration of treatment can be found in the relevant experimental chapters (Chapters 3, 4, 5, 6 and 7).

## **2.4 Follicle culture**

### **2.4.1 Classification of follicle stage**

Follicles were classified as primordial (an oocyte surrounded by a single flat layer of follicular epithelial cells); primary (an oocyte surrounded by a single layer of cuboidal granulosa cells); early preantral (an oocyte surrounded by two to four

granulosa cell layers); preantral (an oocyte surrounded by more than four granulosa cell layers, no antrum formation); antral (follicles containing an antrum that are approximately 400µm in diameter in the rat and less than 2000µm in diameter in the marmoset); and dominant (follicles greater than 600µm in diameter in the rat and greater than 2000µm in diameter in the marmoset). Follicles that had a transparent appearance, an intact basement membrane, even granulosa cell layers, normal morphology and less than 10% pyknosis in the granulosa cells were classified as healthy. Follicles that had greater than 10% pyknosis in the granulosa cells, which is one of the first morphological signs of atresia (Durlinger et al., 2000), were classified as atretic.

#### **2.4.2 Follicle isolation**

Follicles were isolated from the ovaries of 21-day-old Wistar rats (Charles River Laboratories, Tranent, UK) under a laminar flow hood using 27-gauge needles in serum-free endothelial basal medium (EBM)-2 medium (Lonza, Wokingham, UK) supplemented with bovine serum albumin (BSA) (0.1%), L-glutamine (3mM), penicillin (100 IU/ml), streptomycin (0.1mg/ml), transferrin (2.5µg/ml), selenium (4ng/ml), insulin (10ng/ml) and L-ascorbic acid, sodium salt (50µg/ml). Healthy looking preantral/ early antral follicles (170-350 µm), characterized by a transparent appearance, intact basement membrane and even granulosa cell layer were selected for culture, for all experiments. Follicles at these stages were selected as they have undergone recruitment of endothelial cells to the thecal layer and have the highest angiogenic potential.

### 2.4.3 Follicle culture

For all experiments, preantral/early antral follicles were cultured individually in flat-bottomed 12-well plates (Corning Inc., Corning, NY, USA), embedded in the centre of a 20µl droplet of growth-factor reduced Matrigel (BD Biosciences, Bedford, UK). Tissue engineering technologies have recently been applied to the culture of ovarian follicles (Xu et al., 2006) and have shown that a three-dimensional culture system yields favourable results (Pangas et al., 2003). Matrigel is a solubilised basement membrane preparation extracted from Engelbreth-Holm-Swarm mouse sarcoma, a tumour rich in extracellular matrix (ECM) proteins and its main components are structural proteins such as laminin and collagen. Matrigel polymerises to produce a biologically active matrix material resembling the mammalian cellular basement membrane which allows cells to behave as they do *in vivo*. It provides a three-dimensional mechanical support for the follicles as they increase in size, allowing them to maintain an *in vivo*-like morphology. This is highly useful in various studies including *in vitro* and *in vivo* angiogenesis assays as it provides a physiologically relevant environment for cell morphology, biochemical function, migration or invasion; thus allowing vascular outgrowths to develop in an *in vivo*-like manner. Follicles were incubated for 6 days in a sterile humidified air atmosphere with 5% CO<sub>2</sub> at 37°C in 600µl serum-free EBM-2 medium supplemented as described in 2.4.2, and culture medium was refreshed every 48h.

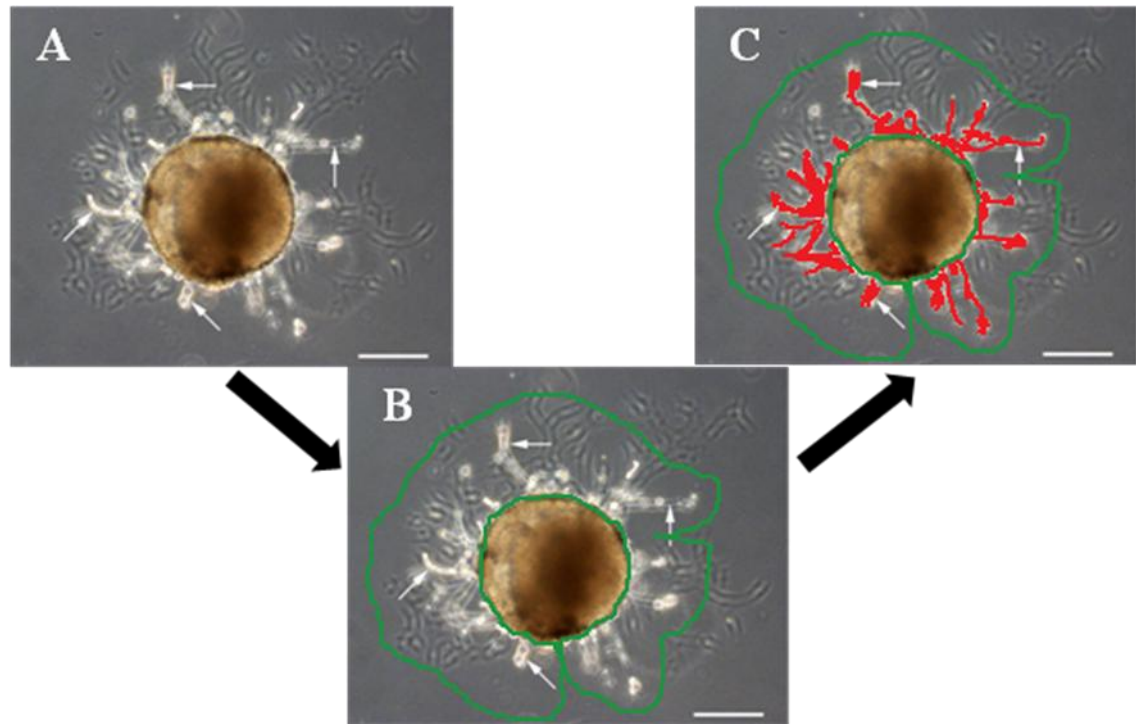
For standard immunohistochemical (IHC) analysis follicles were fixed in 4% neutral buffered formalin (NBF) for 24h before being embedded in paraffin wax. The wax blocks were serial sectioned, and 5µm tissue sections were placed onto BDH



SuperFrost slides (Merck Co., Inc., Poole, UK). Sectioned follicles were stained for haematoxylin and eosin (H&E), activated caspase-3 and Tie-2. For wholemount immunohistochemical analysis follicles were fixed in 4% NBF for 2h and then permeabilised in dH<sub>2</sub>O overnight at 4°C.

#### **2.4.4 Quantification of angiogenesis**

To assess the impact of the treatments on angiogenic sprouting, follicles were examined and photographed directly in the 12-well plates under an inverted light microscope (Axiovert 200), at x10 magnification, with Axiovision image capturing software (Carl Zeiss Ltd, Welwyn Garden City, Hertfordshire, UK) on days 0 and 6 of the culture period. The total area of angiogenic sprouting for each follicle was determined using Image Pro Plus software (Media Cybernetics, Bethesda, MD). This software allows an area of interest to be drawn around the outgrowths (but excluding the follicle), and the sprouts are then highlighted by the software as it allows the selection of light areas against a dark background. The sum of the area of all sprouts was then calculated by the software. The mean area of angiogenic sprouting for each treatment was then compared to the control.



**Figure 2.1 Quantification of angiogenesis**

Diagram illustrates the method of quantifying angiogenesis in the cultured follicles. First the follicle is photographed (A) and then Image Pro-Plus software is used to highlight and measure the area of the sprouts. An area of interest is drawn around the outgrowths, excluding the follicle (B) and then the option to highlight light areas against a dark background is chosen. This highlights the sprouts (C) and then the software measures the area that is highlighted. Area of interest is shown in green and highlighted area to be measured is shown in red. Scale bars represent 200  $\mu\text{m}$ .

## 2.5 Tissue fixation, processing and sectioning

Tissue fixation was needed to stop autolysis, putrefaction, bacterial attack and other undesirable cellular changes. It also keeps the tissue in as close a state as possible to its *in vivo* form. Ovaries were fixed in 4% NBF for 24 h and then transferred to a 70% alcohol ( $\text{C}_2\text{H}_5\text{OH}$ ) solution until processed into paraffin wax. Processing and paraffin wax embedding was carried out by the Unit histology laboratory staff. Tissue was dehydrated through a series of graded alcohols before saturation in paraffin wax. This process was carried out using a 17.5 h automated cycle on a Leica

TP-1050 processor (Leica UK Limited, Milton Keynes, UK). The tissue was then embedded onto plastic cassettes for sectioning.

All tissues were serial sectioned at a thickness of 5µm on to electrostatically charged slides (Merck Co). The electrostatic charging improved the binding of the tissue to the slides. Sections were cut using a hand-operated microtome (Leica UK Ltd) with disposable blades. Sections were floated onto water at 45°C, then transferred onto slides and dried overnight.

## **2.6 Haematoxylin and eosin staining**

H&E staining is used to visualise the tissue for morphometric analysis. Haematoxylin is a purple nuclear stain and eosin is a red cytoplasmic stain. These stains were prepared by Histology staff. For this thesis Harris's mercury free haematoxylin (VWR International Ltd, Hunter Boulevard, Leicestershire, UK) was used and the eosin Y (Richard-Allan, Richland, MI, USA) was prepared by making a 1% solution in distilled water. Sections were dewaxed in two 5min washes of xylene before being taken through graded (100%, 90%, 70%) alcohols and finally taken into water. Once dewaxed, slides were stained in haematoxylin for 5min, washed in water, washed in acid alcohol for 20 seconds, washed in water, washed in Scott's tap water for 30 seconds, washed in water, stained in eosin for 20 seconds and finally washed in water. The slides are then taken through graded (70%, 80%, 95%, 100%) alcohols, washed in xylene and then mounted using Pertex (Cell Path, Hemel Hempstead, UK).

## **2.7 Immunohistochemistry**

Immunohistochemistry is a method used to localise a specific protein within a cell or on the surface of a cell using an antibody raised to an epitope of the protein of interest. This is a useful technique, as it allows a protein to be localised to a specific cell type and often a specific cellular location. The immunohistochemical process involves building up layers of antibodies to increase the available binding sites for the antibody and detection system. Quantification of immunohistochemical staining is possible and is generally based on parameters such as: number of positive cells in a population; the area of staining in a given tissue or structure (in this case the ovarian follicle); or a combination of the two. The advent of image analysis systems and software has increased the efficiency and accuracy of quantification of immunohistochemistry. For all immunohistochemistry negative controls were performed by omitting the primary antibody.

### **2.7.1 Antigen retrieval**

Some antigenic epitopes can be masked during fixation and paraffin wax embedding. Therefore, antigen retrieval by heat treatment (Shi et al., 1991) is necessary to expose those antigens. Slides were dewaxed in xylene, rehydrated through a series of alcohols, and exposed to 0.01M citrate buffer, pH 6, in a Tefal Clypso pressure cooker (Tefal, Essex, UK). Two litres of citrate buffer were heated to boiling point in the pressure cooker and once boiling, racks containing the dewaxed and rehydrated slides were placed into the buffer. The lid was sealed and the slides were heated at high pressure for 5min, starting when full pressure was reached. After pressure was released, the slides remained in the hot buffer for a further 20min before they were

cooled with water and transferred to 0.05M Tris buffered saline (TBS, pH 7.4) for 5min.

### **2.7.2 Blocking non-specific background**

The main cause of non-specific background staining is non-immunological binding of the specific immune sera by hydrophobic and electrostatic forces to certain sites within tissue sections (Kraehenbuhl and Jamieson, 1974). As it is usually the first immune serum that gives rise to the highest non-specific background staining these sites are blocked prior to the application of the primary antibody with immunoglobulins that will not react or interfere with the primary specific antiserum. This is achieved by incubating the sections in normal whole serum from the species in which the secondary antibody was raised. For example, when mouse BrdU was localised using rabbit anti-mouse as a secondary antibody, blocking was performed using normal rabbit serum. All slides were treated with the relevant blocking serum diluted 1:5 in TBS with 20% BSA for 30min at room temperature. Details for each specific antigen are given in Table 1.

### **2.7.3 Immunohistochemical procedure**

Immunohistochemical procedures were performed using an automated incubation method, the Sequenza System (Shandon Scientific, Runcorn, Cheshire, UK). A Sequenza works by capillary action and reduces the volumes of reagents required. Each section was retained within its own Sequenza cassette, which prevents cross-contamination and antibody-antigen complexes are prevented from precipitating onto sections, which would give rise to unwanted background staining.

Unbound antibody was washed from each section before the proceeding step in each protocol. Detection systems vary according to antibody, details are given in Table 1.

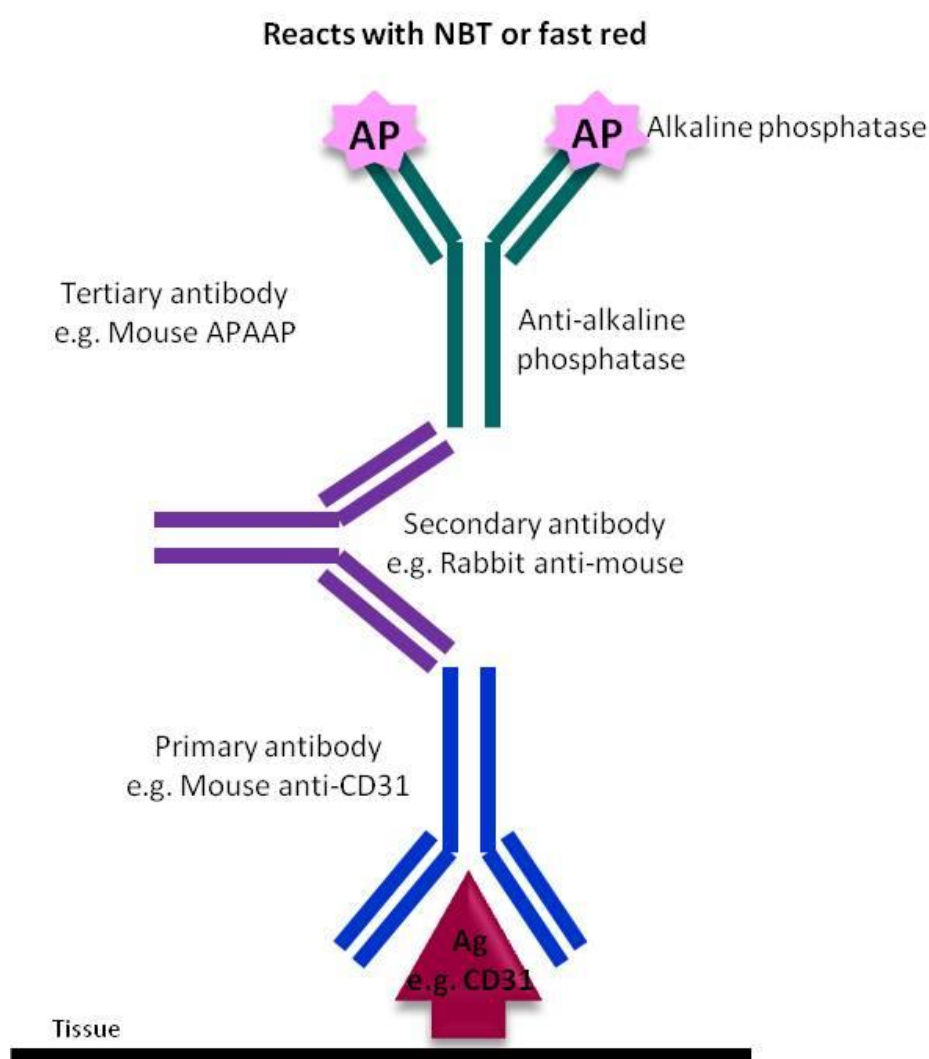
Primary Antibody	Species raised in	Block	Secondary antibody	Tertiary antibody	Detection	Counter stain	Optimisation?
BrdU	Mouse monoclonal	Normal rabbit serum	Rabbit anti mouse	Mouse APAAP	NBT	Yes	No
CD31	Mouse monoclonal	Normal rabbit serum	Rabbit anti mouse	Mouse APAAP	NBT	No	No
CD31/BrdU	Mouse monoclonal Sheep polyclonal	Normal rabbit serum	Rabbit anti mouse Rabbit anti sheep	Mouse APAAP ABC-AP	Fast red NBT	Yes	No
Caspase-3	Rabbit polyclonal	Normal goat serum	Goat anti mouse	Goat anti rabbit peroxidase labelled polymer	DAB	Yes	No
Tie-2	Rabbit polyclonal	Normal goat serum	Goat anti rabbit biotinylated	Streptavidin-HRP	DAB	Yes	Yes

**Table 1 Antibodies and reagents used in immunohistochemistry protocols.**

*Table outline information about the antibodies and detection methods used for IHC performed in this thesis. The majority of the IHC methods had been optimised previously as they are routinely used in our group. However, the protocol for the Tie-2 IHC had to be optimised. The antibody was tested at a range of concentrations and a variety of detection methods and times were tested. It was found that using the antibody at 1:100 and detecting with DAB for 4min gave the best results so these were the condition used.*

Secondary antibodies also varied according to antibody and slides incubated with non-biotinylated secondary antibodies were subsequently incubated with a tertiary alkaline phosphatase-antialkaline phosphatase (APAAP) antibody complex (Cordell et al., 1984). The secondary antibody was applied in excess so that one of its two identical binding sites would bind to the primary antibody, the other to the APAAP

complex, illustrated in Figure 2.2. The APAAP was added, diluted in the appropriate serum block for 40min, before slides were washed in and then transferred to TBS. The APAAP detection method can be used with a variety of substrates giving rise to a variety of colours. The two used in this thesis were nitroblue tetrazolium (NBT), which forms a deep blue/black stain (McGadey, 1970), and fast red used with naphthol AS-MX phosphate sodium, which gives a bright red stain.



**Figure 2.2 Illustration of the APAAP detection system**

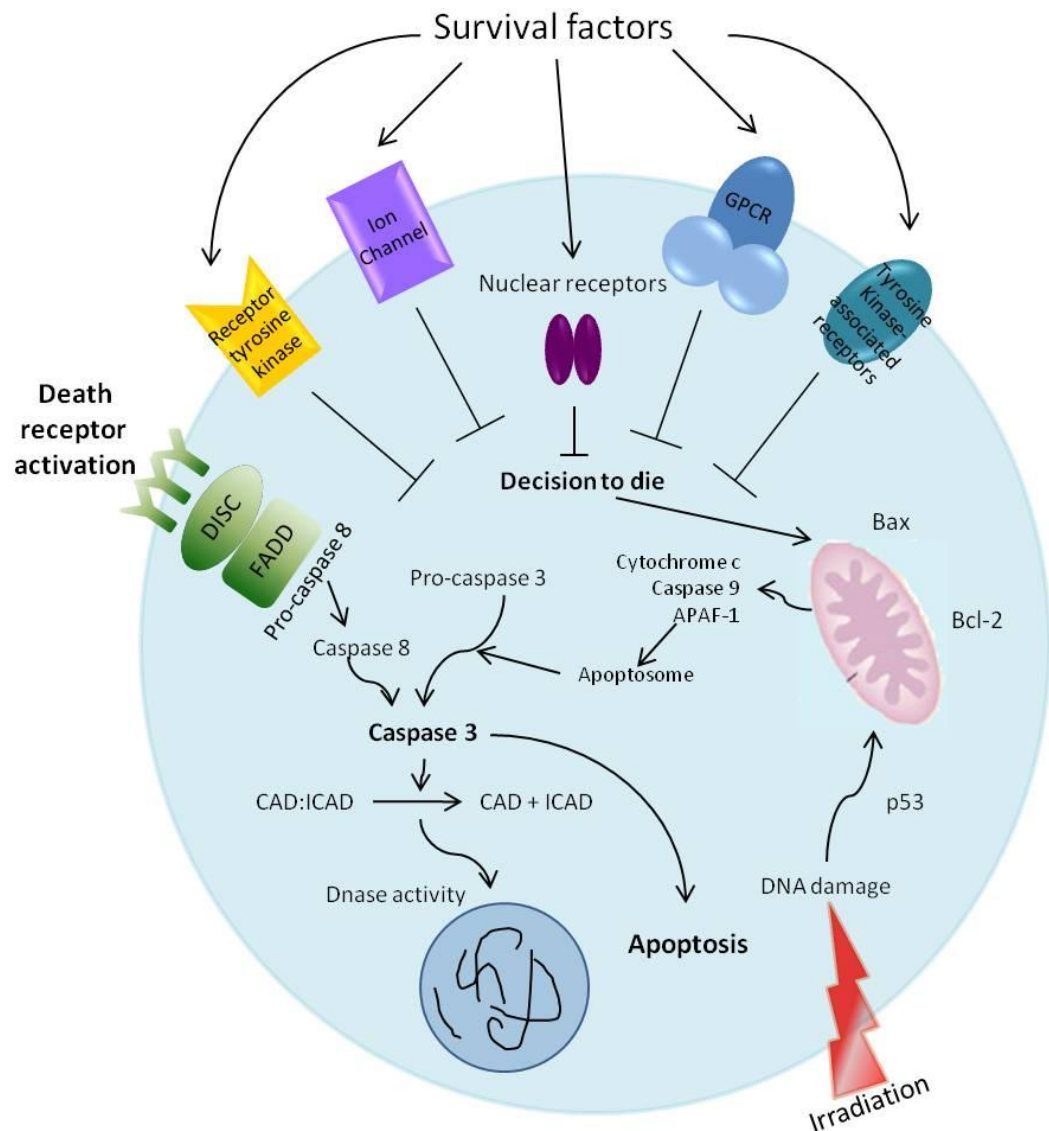
*Diagram showing a primary antibody binding to the specific antigen. The secondary antibody binds to the primary antibody and also to the APAAP complex. Staining can then be detected using either NBT or fast red.*

## **2.7.4 Specific immunohistochemistry techniques in the rodent**

### **2.7.4.1 Caspase-3 (apoptotic cells)**

Although other techniques for the detection of apoptosis, such as TdT-mediated dUTP-biotin nick end labelling (TUNEL), are useful, they often have issues with specificity and interpretation of the results (Allen et al., 1997). Therefore, the development of antibodies to more generally distributed apoptotic markers, such as activated caspase-3, are preferred for the detection of apoptosis (Barrett et al., 2001). The caspases are a family of cysteine proteases that are essential for apoptosis (Thornberry and Lazebnik, 1998). There are two types of apoptotic caspases, initiator caspases (caspases 2, 8, 9, 10 and 12) and effector caspases (caspases 3, 6 and 7) (Thornberry and Lazebnik, 1998, Lavrik et al., 2005). The initiator caspases are closely coupled to pro-apoptotic signals such as FasL (Fas ligand), tumour necrosis factor (TNF) and DNA damage. Caspases are synthesised as inactive pro-caspases and are converted to active caspases by cleavage at specific aspartate residues or by autocatalysis due to either the removal of inhibitors or the binding of co-factors (Cohen, 1997). These activated initiator caspases then activate the effector caspases which have many downstream effects that result in programmed cell death, including the inactivation of cytoprotective proteins such as the ICAD/CAD complex and Bcl-2 (Thornberry and Lazebnik, 1998). The caspases have different roles within the apoptotic cascade and caspase-3 is one of the predominant caspases that is activated in cells undergoing programmed cell death (Faleiro et al., 1997). A diagram of the apoptotic cascade is provided in Figure 2.3.



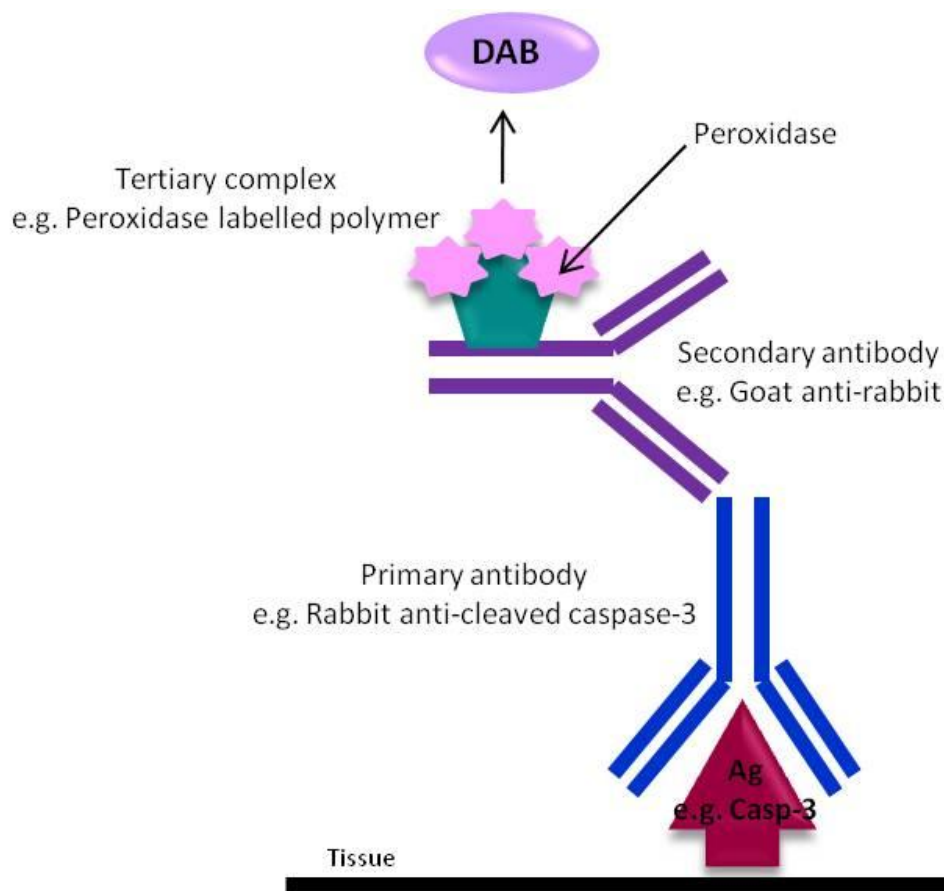


**Figure 2.3 Schematic overview of the apoptotic process.**

There are several factors involved in regulating the 'decision to die'. In granulosa cells examples of survival factors include the gonadotrophins FSH and LH, the steroids progesterone ( $P_4$ ) and estrogen ( $E_2$ ), and other factors including growth hormone, insulin-like growth factor I (IGF-I), bFGF and insulin. Pro- and anti-apoptotic signals interact in the mitochondria and when pro-apoptotic activity is greater than anti-apoptotic activity molecules are released. These molecules activate the caspase cascade and apoptosis occurs. In addition, apoptosis can be induced via DNA damage and activation of the death receptor pathway, as shown on the diagram. GPCR – G-protein coupled receptor, DISC – Death-inducing signalling Complex, FADD – Fas-associated protein with death domain, CAD – Caspase-activated DNase, ICAD – Inhibitor of CAD, APAF-1 – Apoptotic protease activating factor-1. (Figure adapted from (Markstrom et al., 2002))

For the purposes of this thesis, a polyclonal antibody to cleaved (activated) caspase-3 (Asp175; New England Biolabs, Hitchin, UK) was used to determine the localization and changes in the number of dying cells and a peroxidase labelled polymer was used with 3,3' diaminobenzidine (DAB) was used for immunohistochemical visualisation. DAB yields a crisp, insoluble, dark brown reaction product (Graham and Karnovsky, 1966), a diagram illustrating this technique is provided in Figure 2.4. As endogenous peroxidase in animal tissues can react with DAB and cause a blurring of the resulting stain, slides were treated with saturating amounts of hydrogen peroxide to irreversibly inactivate these endogenous peroxidases.

Therefore, immediately after antigen retrieval, endogenous peroxidase activity was quenched by a 5min incubation in peroxidase block (EnVision HRP kit, Dako), and then the slides were washed three times with TBS for 5min and then blocked in normal goat serum (NGS, diluted 1:5 in TBS containing 5% BSA), for 30min. The sections were incubated overnight at 4°C with activated caspase-3 antibody (1:100 in NGS). Slides were washed in TBS and incubated with labelled polymer-horse radish peroxidase (HRP) as secondary antibody (rabbit EnVision kit) for 30min. Slides were washed and transferred to TBS before the tissue sections were dried around and DAB substrate added. Staining was produced after approximately 5min. Reactions were stopped in tap water and sections were counterstained in haematoxylin for approximately 10 seconds before being dehydrated through graded alcohols, cleared and mounted using Pertex.



**Figure 2.4 Illustration of EnVision DAB/Chromagen immunohistochemical detection.**

*Diagram showing a primary antibody binding to its specific antigen. The secondary antibody that binds to the primary is biotinylated to allow binding of the tertiary complex. Coloured substrates are then used for visualisation of detection, e.g. DAB.*

#### 2.7.4.2 Tie-2

To determine the localisation of Tie-2 in the animal models used in this thesis, IHC was performed. Immediately after antigen retrieval, endogenous peroxidase activity was quenched by a 30min incubation in peroxidase block (30ml 30%  $\text{H}_2\text{O}_2$  and 270ml methanol), the slides were washed three times with TBS for 5min and then blocked in NGS, for 30min. The slides were blocked with streptavidin for 15min followed by three TBS washes, blocked with biotin for 15min followed by three TBS

washes and then the sections were incubated overnight at 4°C with an antibody to Tie-2 (1:100 in NGS, sc-324 Santa Cruz Biotechnology, Heidelberg, Germany). Slides were washed three times in TBS-Tween and incubated with goat anti-rabbit biotinylated secondary antibody (1:500 in NGS). Following three TBS-Tween washes, slides were incubated in streptavidin labelled polymer-HRP as tertiary antibody (1:1000 in TBS) for 30min. Slides were washed and transferred to TBS before the tissue sections were dried around and DAB substrate added. Staining was produced after approximately 5min. Reactions were stopped in tap water and sections were counterstained in haematoxylin for approximately 10 seconds before being dehydrated through graded alcohols, cleared and mounted using Pertex.

## **2.8 Wholemout Immunohistochemistry**

To confirm the endothelial nature of the angiogenic sprouting, a sample of follicles was analyzed by wholemount IHC for incorporation of various endothelial cell markers. Negative controls were performed for all immunohistochemistry by omitting the primary antibody.

### **2.8.1 Immunohistochemical procedure**

Follicles were fixed for 2h in 4% NBF and permeabilised in dH<sub>2</sub>O overnight at 4°C. Follicles were washed in between each treatment step in tris-buffered saline-Tween (TBS-Tween) for 5min 3-5 times and all incubations were carried out at room temperature unless stated otherwise.

**2.8.1.1 Dil-Ac-LDL**

1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate (Dil-Ac-LDL) (20µg/ml, Serotec, Oxford, UK) is a fluorescent-labelled acetylated low-density lipoprotein is taken up by macrophages and endothelial cells via the “scavenger” metabolic pathway (Voyta et al., 1984). To confirm the endothelial nature of the angiogenic sprouting Dil-Ac-LDL was added to the culture medium on day 4, and outgrowths were assessed for uptake of the molecule on day 6 via confocal fluorescence microscopy at 568nm.

**2.8.1.2 CD31**

Platelet-endothelial cell adhesion molecule (PECAM-1/CD31) is a commonly used endothelial cell marker (Ilan et al., 2000) and is a 130-kDa glycoprotein belonging to the Ig superfamily of cell adhesion molecules. CD31 expression is restricted to cells of the vascular system platelets, monocytes, neutrophils, selected T cells, and endothelial cells (Newman, 1997, Newman, 1999). It has previously been shown that CD31 knockout mice suffer from prolonged bleeding times after surgical insult, which is at least in part due to disrupted endothelial-platelet interactions (Mahooti et al., 2000). In addition to the role of CD31 in endothelial cell adhesion, there is growing evidence that CD31 is able to transduce signals that suppress endothelial cell apoptosis (Noble et al., 1999, Newman and Newman, 2003).

For CD31 IHC endogenous peroxidase activity was quenched with hydrogen peroxidase at 1% in phosphate-buffered saline (PBS) for 30min, then blocked in NGS for 30min, and then incubated with an antibody to CD31 (1:300 in NGS,

550300 BD Biosciences Pharmingen, Oxford, UK) overnight at 4°C. Follicles were incubated with goat anti-mouse peroxidase with Fab fragments (1:500 in NGS) and kept in the dark before incubating with Tyramide TSA-Plus Cy3 System (1:50, Perkin Elmer Life Sciences, Beaconsfield, UK) for 10min. After washing, labelling was detected using confocal fluorescence microscopy at 568nm (red channel to show CD31 stained cells).

#### **2.8.1.3 CD34**

CD34 is a cell surface glycoprotein and acts as a cell adhesion factor. It is expressed on both endothelial progenitor cells and endothelial cells and is therefore a marker for endothelial cells (Muller et al., 2002, Pusztaszeri et al., 2006). For CD34 IHC follicles were blocked in normal donkey serum (NDS, diluted 1:5 in TBS containing 5% BSA) for 1h, and then incubated with an antibody to CD34 (1:100 in NDS, sc-7045 Santa Cruz) overnight at 4°C. After washing, follicles were incubated with donkey anti-goat-AlexaFluor555 (1:200 in PBS) for 1h and then labelling was detected using confocal fluorescence microscopy at 546nm (red channel to show CD34 stained cells).

#### **2.8.1.4 $\alpha$ SMA**

Alpha-smooth muscle actin ( $\alpha$ SMA) is a differentiation marker of smooth muscle cells and is also present in myofibroblasts and pericytes.  $\alpha$ SMA expressing cells have important roles in angiogenesis as myofibroblasts induce wound contraction during normal wound healing (Grinnell, 1994, Powell et al., 1999, Tomasek et al., 2002). In addition, pericytes are recruited to developing blood vessels during angiogenesis to support and stabilise the new vessels. Therefore, the expression of  $\alpha$ SMA by new

blood vessels is an indicator of blood vessel maturity. For  $\alpha$ SMA IHC follicles were blocked in NGS and then incubated with an antibody to  $\alpha$ SMA primary antibody (1:500 in NGS, A2547 Sigma Aldrich) at 4<sup>0</sup>C overnight. After washing, follicles were incubated with goat anti-mouse-AlexaFluor488 (1:200 in PBS) for 1h and then labelling was detected using confocal fluorescence microscopy at 488nm (green channel to show  $\alpha$ SMA labelled cells).

#### **2.8.1.5 CD31/ $\alpha$ SMA Dual**

For the CD31/ $\alpha$ SMA dual IHC the method for CD31 IHC was followed as above. After Tyramide staining follicles were incubated with the  $\alpha$ SMA primary antibody (1:500 in NGS) at 4<sup>0</sup>C overnight. After washing, follicles were incubated with goat anti-mouse-AlexaFluor488 (1:200 in PBS) for 1h and then labelling was detected using confocal fluorescence microscopy at 488nm (green channel to show  $\alpha$ SMA labelled cells) and 568nm (red channel to show CD31 stained cells) simultaneously.

#### **2.8.1.6 TdT-mediated dUTP-biotin nick end labelling (TUNEL)**

To provide another method for investigation of the effect of TSP-1 on apoptosis, whole follicles from control and TSP-1 treatment groups were subjected to TUNEL analysis in order to detect proportions of apoptotic cells. At the end of the culture period, follicles were transferred to 24-well culture plates (Corning Inc.) and then washed (10 min, PBS, 37<sup>0</sup>C). Cultured follicles were permeabilised and prefixed (40 min; 0.5% Triton-X100, 0.25% paraformaldehyde in PBS at 37<sup>0</sup>C), and then fixed (30 min; 4% paraformaldehyde in PBS at room temperature), washed in PBS (2x 10 min) and stored (0.02% NaN<sub>3</sub> in PBS, 48<sup>0</sup>C). Follicles were washed and equilibrated to room temperature with PBS (20 min; 2x 10 min). Samples were then incubated

with proteinase K (Roche, Welwyn Garden City, Hertfordshire, UK) (30 min, 17.1  $\mu\text{g ml}^{-1}$  in PBS, 37°C), refixed at room temperature (20 min, 3% paraformaldehyde in PBS) and washed (20 min, 0.01% Triton-X100 in PBS; 2x 10 min PBS). A preincubation with TdT buffer (10 min, 30 mmol Tris-HCl  $\text{l}^{-1}$ , pH 7.2, 140 mmol sodium cacodylate  $\text{l}^{-1}$ , 1 mmol cobalt chloride  $\text{l}^{-1}$ ) was carried out before incubation with TUNEL reaction mixture (2.5 h, 37°C in the dark) from an in situ cell death detection kit, Fluorescein (Roche), which was prepared according to the manufacturer's instructions. Still in the dark, follicles were washed in PBS (2x 10 min) and incubated with propidium iodide (1 h, 2.5  $\mu\text{g ml}^{-1}$ , room temperature) in bovine pancreatic RNase A (Roche). Samples were washed (20 min, 0.01% Triton-X100 in PBS; 2x 10 min PBS) and stored in PBS at 4°C in the dark.

### **2.8.2 Detection of Wholemount immunohistochemistry**

Follicle sections were analysed using the Leica TCSNT 710 confocal system (Leica Microsystems, Milton Keynes), equipped with a number of standard objective lenses. The pinhole size was 1 Airy unit. A z-stack was taken through the centre of each follicle using the first/last method and a plan-neofluor x10/ 0.3-n.a. objective lens. The acquisition parameters and number of slices to be taken were determined using the Nyquist calculator on the CALM website. Simultaneous scans at 488nm (the green channel) and 546nm (the red channel) were taken and then the z-stacks were converted into three dimensional projections using the Zen software.



## **2.9 Granulosa cell culture**

21-day-old female Wistar rats (Charles River Laboratories) were used as described above. Two animals were used for each experiment and experiments were done in triplicate. Animals were killed by asphyxiation with CO<sub>2</sub> and the ovaries removed.

### **2.9.1 Isolation of granulosa cells**

Granulosa cells were isolated by puncturing follicles with a 27-gauge needle and gently expelling the cells into culture medium. Pooled cells were centrifuged, re-suspended in fresh M199 medium (Gibco, Invitrogen Life Technologies, Inc.), Paisley, UK) containing 0.1% BSA, 2 mmol L-glutamine, 50µg/ml streptomycin and 50 IU/ml penicillin and their viability assessed by counting a Trypan blue-stained preparation in a haemocytometer. Cell viability was 30-40%.

### **2.9.2 Culture of granulosa cells**

To measure caspase activity tissue culture grade 96-well plastic plates (Corning Inc.) were precoated with 0.1ml donor calf serum (Lonza) and washed twice with PBS (0.1ml) before inoculating with 0.1ml medium containing  $1 \times 10^5$  cells/ml. Following overnight preincubation in a sterile humidified air atmosphere with 5 % CO<sub>2</sub> at 37°C, prewarmed medium containing varying doses of the compound of interest was added to triplicate wells and the incubation continued for 48h.

To measure expression of various factors by qRT-PCR tissue culture grade 6-well plastic plates (Corning Inc.) were precoated with 0.5ml donor calf serum (Lonza) and washed twice with PBS (0.5 ml) before inoculating with 1.5ml medium containing 3

$\times 10^5$  cells/ml. Following overnight preincubation in a sterile humidified air atmosphere with 5 % CO<sub>2</sub> at 37°C, 1.5ml prewarmed medium containing varying doses of the compound of interest was added to triplicate wells and the incubation continued for 48h.

To measure expression of various factors by western blotting, tissue culture grade 6-well plastic plates (Corning Inc.) were precoated with 0.5ml donor calf serum (Lonza) and washed twice with PBS (0.5ml) before inoculating with 1.5ml medium containing  $3 \times 10^5$  cells/ml. Following overnight preincubation in a sterile humidified air atmosphere with 5 % CO<sub>2</sub> at 37°C, 1.5ml prewarmed medium containing varying doses of the compound of interest was added to triplicate wells and the incubation continued for 96h with medium refreshed every 48h.

### **2.9.3 Caspase-3/7 assay**

To measure caspase-3 and -7 activities in isolated granulosa cells, the Caspase-Glo 3/7 assay was performed according to the manufacturer's instructions (Promega UK Ltd., Southampton, UK).

Treatments were carried out in triplicate and experiments were repeated three times. As serum can generate a background caspase activity signal, two additional wells contained cell culture medium and carrier solution without any cells. To ensure reagent activity and signal detection were working effectively an additional two wells contained 50ng/ml TNF $\alpha$ , which was used as a positive control.

After 48h, the granulosa cells were allowed to equilibrate to room temperature and 100µl of blank, negative control cells, positive control cells or treated cells were added to each well of a white-walled 96-well plate (Corning Inc.). Caspase-Glo 3/7 reagent was added directly to the cells in culture medium in a 1:1 ratio. The well contents were then mixed for 30secs and incubated at room temperature for up to 3h. This resulted in cell lysis, followed by caspase cleavage of the substrate and generation of a glow-type luminescent signal, produced by luciferase. Luminescence was measured using a FLUOstar OPTIMA microplate reader (BMG Labtech Ltd., Aylesbury, UK). Luminescence was directly proportional to the amount of caspase activity present. The value for the no cell control was subtracted from the experimental values. The experiment was repeated three times to reduce the possible effects of biological variability.

#### **2.9.4 RNA extraction and cDNA synthesis**

At the end of the culture period, RNA was extracted for analysis, using the RNeasy micro-kit (Qiagen, West Sussex, UK), following the manufacturer's instructions. Cells were lysed in 350µl RLT buffer, collected in a Safe-Lock tube (Eppendorf Eppendorf UK Limited, Cambridge, UK) and vortexed. A volume of 350µl of 70% ethanol was added to the lysate and mixed well by pipetting. The sample was then transferred to an RNeasy MinElute spin column and centrifuged at  $\geq 8000 \times g$  for 15secs. A volume of 350µl wash buffer RW1 was added to the spin column and centrifuged for 15secs at  $\geq 8000 \times g$ . On-column digestion of DNA was then performed by addition of DNase1 (Qiagen) which was incubated at room temperature for 15min. DNase1 was then removed by a further wash with wash

buffer RW1. Subsequently 500µl of buffer RPE containing ethanol, to wash the RNA, was added to the column and centrifuged at  $\geq 8000 \times g$  for 15secs. A volume of 500µl of 80% ethanol was added to the spin column and centrifuged for 2min at  $\geq 8000 \times g$  to wash the spin column membrane. The spin column was then dried by centrifugation at full speed for 5min with the spin column lids open. This longer centrifuge ensured all residual ethanol was removed from the columns as residual ethanol can interfere with downstream reactions. The RNA, which had remained stuck to the column throughout, was eluted in 14µl of RNase-free water and centrifuged for 1min at  $\geq 8000 \times g$ . The eluate was collected in new microcentrifuge tubes.

#### **2.9.4.1 cDNA Synthesis**

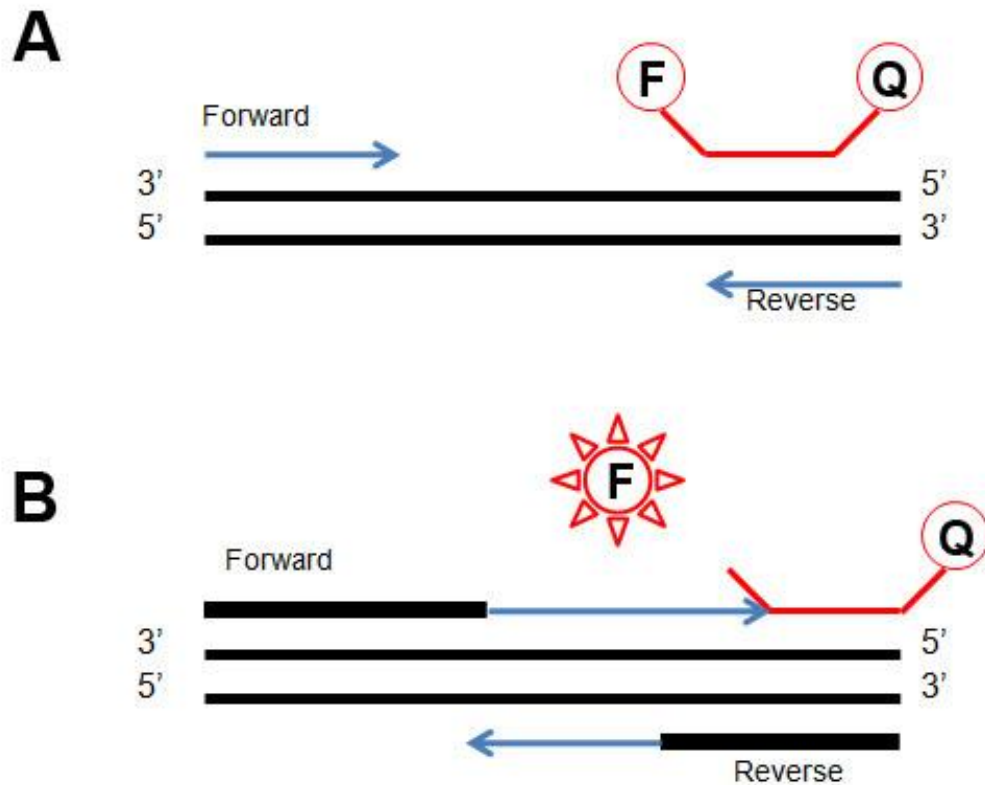
The concentration of the RNA was determined prior to cDNA synthesis using the Nanodrop 1000 (Thermo Scientific, Loughborough, UK). This machine automatically calculates the concentration ( $A_{260}$ ) and quality ( $A_{260/280}$ ) of RNA. RNA was reverse-transcribed using the Vilo cDNA synthesis kit (Invitrogen) in a reaction mixture (total vol. 20µl) containing 4µl 5x Vilo Reaction Mix, 0.25µl 10x Superscript Enzyme Mix, 100ng/µl RNA and topped up to 20µl with RNase free  $H_2O$ . The cDNA was then amplified in a cyclic process of DNA denaturation, specific primer annealing and primer extension using the Peltier Thermal Cycler-200 DNA Engine Cycler (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) under the conditions 25°C for 10 min, 42°C for 60min and 85°C for 5min. This results in the primer specified cDNA fragment being exponentially increased. Genomic DNA

contamination was excluded in each PCR using a control for each sample that was generated in the absence of reverse transcriptase.

## **2.9.5 Taqman quantitative RT-PCR**

### ***2.9.5.1 Principle of Taqman reaction***

Taqman quantitative RT-PCR was used to accurately quantify mRNA of genes of interest. Taqman probes have the fluorophore FAM attached to the 5' end and a quencher dye (TAMRA for Universal Probe library probes, Non-fluorescent for Assay-On-Demand probes) attached to the 3' end. The 18S probe used in this thesis used the VIC fluorophore to distinguish it from the gene of interest. After annealing to the gene of interest Taq polymerase extends the primer 5' to 3' and displaces FAM. This leads to a separation between the fluorophore and the quencher, leading to an increase in fluorescence which can be measured. As the PCR reaction is exponential, the original amount of cDNA in the sample, and therefore RNA, can be quantified by measuring the amount of fluorescence (Figure 2.5).



**Figure 2.5 Principle of Taqman reaction**

A: Specific primers and probe anneal to the gene of interest. Taq polymerase begins to extend the primers from 5' to 3'

B: Taq polymerase extends the primer to displace the fluorophore FAM (F) from the TAMRA quencher (Q). This results in an increase in fluorescence which can be measured and which increases as the PCR reaction continues exponentially.

#### 2.9.5.2 Taqman reaction

Expression of Tie-2, Ang-2 and 18S rRNA mRNA was measured in cDNA samples from granulosa cells cultured in the presence or absence of test compounds, in triplicate. Negative controls included RT- samples and nuclease-free water.

Expression was measured using an ABI 7900HT Real-Time PCR System (Applied Biosystems, Warrington, UK). Oligonucleotide primers for PCR were designed to

amplify Tie-2 using the Universal Probe library (Roche) and custom ordered from MWG (Eurofins MWG Operon, Ebersberg, Germany) – forward primer GCAAATGTCCAAAGAAGAATG, reverse primer TGAACAGCTGGTTCTTCTCTCA. The corresponding probe, according to the Universal Probe library was ordered from Roche (Probe #97). Primers were designed with the following features:

- No multiple repeats of a nucleotide, particularly guanosine
- A melting temperature of 58-60°C that was equal between the forward and reverse primers
- Primers that were intron spanning, to avoid amplifying genomic sequences
- Primers that were as close together as possible, without overlapping and that were 50-150bp in length
- Primers that amplified segments of the sequence that displayed low homology with other genes in the same family

PCR for Ang-2 was performed using Assays on Demand Gene Expression Products (Agpt2; TaqMan assay ID: Rn01756774\_m1) (Applied Biosystems).

For the PCR for Tie-2, 1.5µl cDNA was amplified in a reaction mixture (total vol, 15µl) containing 7.5µl 2x Express Mastermix (Invitrogen), 0.1125µl 18S primer/probe mix (Invitrogen), 0.15µl each of forward and reverse primers (MWG), 0.15 of the target probe (probe 97 – Roche Universal Probe Library) and 5.4375µl double distilled H<sub>2</sub>O. This mix was added in triplicate to a 96-well MicroAmp fast optical reaction PCR plate (Applied Biosystems). Reactions were incubated for 5min

at 45°C followed by 5 min at 95°C, followed by PCR cycling for 40 cycles of 15sec at 95°C and 1min at 60°C.

For the PCR for Ang-2, 1.5µl cDNA was amplified in a reaction mixture (total vol, 15µl) containing 7.5µl 2x Express Mastermix, 0.225µl 18S primer/probe mix, 0.75µl of the 20x Assay-on-demand primer probe mix and 5.025µl double distilled H<sub>2</sub>O. This mix was added in triplicate to a 96-well MicroAmp fast optical reaction PCR plate. Reactions were incubated for 5min at 45°C followed by 5 min at 95°C, followed by PCR cycling for 40 cycles of 15sec at 95°C and 1min at 60°C.

To determine change in specific gene expression in the follicles, the levels of expression of specific mRNAs in each sample were quantified using the  $2^{-\Delta\Delta Ct}$  method. To ensure the integrity of these results and to account for possible variation due to differing amounts of RNA and pipetting errors, an endogenous housekeeping gene, 18S, was used as an internal standard to ensure that the genes of interest were not regulated under any of the culture conditions tested. This gene has been identified as an appropriate housekeeping gene for use in quantitative PCR studies (Thellin et al., 1999, Schmittgen and Zakrajsek, 2000).

First the expression of the gene of interest is normalised to that of the housekeeping gene by subtraction of the threshold cycle (Ct; the cycle number at which the gene is termed  $\Delta Ct$ ). This assumes that the primers and probes of 18S and those of the gene of interest are working at similar efficiencies (see 2.9.5.3). To determine change in specific gene expression in the follicles, the levels of expression of specific mRNAs



in each sample were quantified using the  $2^{-\Delta\Delta C_t}$  method. This is derived from the equation describing exponential amplification of PCR:

$$X_n = X_0(1 + E_x)^{C_{t,x}}$$

Where  $X_n$  is the number of templates at cycle  $X$ ,  $X_0$  is the number of template copies at initiation,  $E_x$  is the efficiency of target amplification and  $n$  is the number of cycles.

As the  $\Delta\Delta C_t$  of the control sample is subtracted against itself, its final value after applying  $2^{-\Delta\Delta C_t}$  is 1. Therefore, all other samples are expressed as a percentage increase relative to this.

### **2.9.5.3 Primer and probe validation**

Increasing dilutions (from 1:10 to 1:1000) of cDNA were used to generate standard curves, for Tie-2, Ang-2 and 18S, of the number of cycles needed to yield a fluorescent signal above background against the log of relative concentration. The resulting slope of the curve is a measure of the efficiency of the PCR reaction (Hartley et al., 2002). The slopes of the standard curves are indicative of the efficiency of the PCR reaction and were all close to -3.3 (equivalent to 100% PCR efficiency or two-fold amplification per cycle), allowing quantification using the  $2^{-\Delta\Delta C_t}$  method.

### **2.9.6 Protein extraction**

At the end of the culture period (when granulosa cells were above 50% confluent), protein was extracted for analysis. Medium was removed from cells and 120µl of lysis buffer (1ml glycerol, 50µl 1M HEPES pH7.4, 40µl 0.5M EDTA pH8.0, 100µl NP40, 0.146g NaCl, 300µl PMSF, 300mM NaVO<sub>4</sub>, 30µl Leupeptin, made up to 10ml

with dH<sub>2</sub>O) was added at 4°C to each well of a 6-well plate. Cells were scraped off the plates and transferred to a chilled 1.5ml eppendorf and then centrifuged at  $\geq 10,000 \times g$  for 10min at 4°C. A volume of 100µl of supernatant was removed and mixed with 100µl 2xLSB (40ml 10% SDS, 12.5ml 1M Tris pH6.8, 5ml glycerol, 3.75ml dH<sub>2</sub>O, a small amount of bromophenol blue powder and just before use 7µl/ml β-mercaptoethanol was added) to give the cytoplasmic fraction of the cell extract. To get the nuclear fraction 100µl of lysis buffer was added at 4°C to pellet and centrifuged at  $\geq 10,000 \times g$  for 10min at 4°C. A volume of 100µl of supernatant was removed, mixed with 100µl 2xLSB and sonicated to disrupt the nuclei. Samples were stored at -80 °C, until use.

### **2.9.7 Determination of protein concentration**

To ensure even loading of protein on western gels the concentration of the protein samples was determined using the Bradford protein assay. Standards of 1, 2, 3, 5, 7.5, 10, 15, 20µl BSA were made up in eppendorfs to 800µl with PBS and 5µl of the extracted protein was added to 795µl PBS for each sample, with 200µl of Bradford dye added to each tube. The samples were then read on a lightwave spectrophotometer at 595nm absorbance. The blank sample of 800µl PBS and 200µl Bradford dye was read first, then the BSA standards followed by the extracted protein samples. The readings for the BSA standards were plotted using Excel and a trend line added to the data points. The equation of this trendline was used to work out the concentration of the samples as y is protein concentration and x is absorbance of the sample. If you put the value for x into the equation you can work out the concentration of your sample and then divide by 5 to get the concentration in µg/µl.

### **2.9.8 Western blotting**

Western blot analysis was conducted to investigate the expression of various proteins in granulosa cells. For each gel 5µl of MWt ladder and 10µl (approx 10µg protein) of sample was loaded and resolved on 4–20% Tris-glycine gels (NOVEX, Invitrogen) and transferred onto a polyvinylidene difluoride fluorescent (PVDF-fl) membrane (Millipore Corp., Watford, UK) using the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). Membranes were blocked for 1h at 25°C in Rockland Near Infra Red blocking buffer (Tebu-Bio, Peterborough, UK) and incubated overnight at 4°C in blocking buffer containing rabbit anti-phospho-p42/44 ERK (1:1000, 4370 Cell Signalling Technologies, New England Biolabs, Hertfordshire, UK), rabbit anti-phospho-p38 (1:500, 9215 Cell Signalling Technologies), FOXO1 (1:1000, 2880 Cell Signalling Technologies), pTie-2 (1:1000, AF2720 R&D Systems), HIF1α (1:500, sc-10790 Santa Cruz) VEGF (1:200, sc-507 Santa Cruz), Tie-1 (1:1000, sc-342 Santa Cruz), Tie-2 (1:1000, sc-324 Santa Cruz) or Ang-2 (1:1000, AB3121 Millipore) and β-actin (1:1000, sc-1616 Santa Cruz). After washing in PBS containing 0.5% Tween, membranes were incubated for 1h at 25°C in the dark in donkey anti goat IgG conjugated to IRDYE 800 (605-732-125 Tebu-Bio) and donkey anti rabbit IgG conjugated to AlexaFluor 680 (A10043 Invitrogen) both at a dilution of 1:5000 in blocking buffer. Membranes were washed and proteins visualized and quantified using an Odyssey Infrared Imaging System (Li-Cor Bioscience, Cambridge, UK). Sample loading was standardised to expression of β-actin.

## 2.10 RNA knockdown

RNA knockdown was performed to investigate the importance of various genes in the angiogenic cascade. First experiments were carried out to confirm that lentiviral delivery would be effective in this cell type and also to establish the conditions required for efficient transfection, by culturing granulosa cells that were isolated as described above (2.9.1 and 2.9.2) with green fluorescent protein (GFP)-expressing lentivirus (see Chapter 6 for details). The efficiency of transfection was then determined by culturing granulosa cells with lentivirus containing miRNA for LacZ at 1 virus/cell. Granulosa cells were cultured for 48h before being cultured with the LacZ-expressing lentivirus for 8h. After 8h the lentivirus was removed and cells were fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 5min at room temperature. Cells were washed and then stained with X-gal staining solution (23ml 0.5M NaH<sub>2</sub>PO<sub>4</sub>, 77ml 0.5M Na<sub>2</sub>HPO<sub>4</sub>, 1.06g potassium ferrocyanide, 0.82g potassium ferricyanide, 5ml 1% deoxycholate, 5ml 2% NP40, 1ml 1M MgCl<sub>2</sub>, made up to 500ml with dH<sub>2</sub>O; 2ml X-gal added per 100ml staining solution) overnight at 37°C. To assess the efficiency of transfection the total number of cells and total number of X-gal stained cells (blue) were counted in 10 random fields of view and the averages were used to estimate the transfection efficiency using the calculation:

$$\frac{\text{Total number of blue cells}}{\text{Total number of cells}} \times 100 = \% \text{ transfection}$$

To determine the level of GFP-expressing cells the total number of cells and total number of GFP-expressing cells (green) were counted in 10 random fields of view and the averages were used to estimate the percentage of cells expressing GFP.

RNA knockdown was performed to investigate the importance of Tie-1, Tie-2 and Ang-2 in the angiogenic cascade and follicle health. Transient transfection of granulosa cells were performed using microRNA (miRNA) constructs. Oligonucleotides encoding miRNA constructs for Tie-1, Tie-2 and Ang-2 were obtained from Invitrogen and also designed by the in-house Biomolecular Core Facility. The in-house Biomolecular Core Facility then inserted these miRNA constructs into a cloning vector, prior to being inserted into GFP-expressing lentivirus.

After the initial experiments to determine optimal conditions for efficient transfection (see Chapter 6), granulosa cells were isolated as described above (2.9.1 and 2.9.2) and cultured on tissue culture grade 12-well plastic plates (Corning Inc.) which were precoated with 0.5ml donor calf serum (Lonza) and washed twice with PBS (0.5 ml) before inoculating with 0.5ml M199 medium containing 0.1% BSA, 2 mmol L-glutamine, 50µg/ml streptomycin and 50 IU/ml penicillin, containing  $1.5 \times 10^5$  cells/ml for 48h before being cultured with the GFP-expressing lentivirus containing the miRNA construct at 10 viruses/cell, for 8h. After 8h the lentivirus was removed and replaced with fresh medium for 24h. The cells were then treated with 0.5ml M199 medium containing the compound of interest for 48h before protein or RNA was extracted from the cells to allow quantification of expression of genes of interest.

## **2.11 Marmoset Tissue**

### **2.11.1 Marmoset husbandry**

All marmosets were housed in a primate facility located at the R. V. Short Building, Bush Estate, Edinburgh. Staff at the centre were responsible for the care and maintenance of the animals. Common marmoset monkeys were housed in rooms kept at between 20°C and 25°C, which were artificially lit between the hours of 07:00 and 19:00. The animals were housed in cages measuring 1.15m x 1.1m x 0.6m, containing larch branches and a nest box, and the floor was filled with wood chippings to allow foraging.

The animals were fed daily with a selection of fruit and SDS Mazuri (E) primate diet pellets. In addition, they were given high protein porridge with multivitamin supplements three times per week and water was continuously available. Adult females with a body weight of approximately 350g, and who had regular ovarian cycles based on their progesterone cycling, were housed together with a younger sister or prepubertal female, unless otherwise stated. Blood samples were collected three times per week by femoral venepuncture without anaesthesia, while the animals were held in a restraining device (Hearn et al., 1978). Syringes were sealed and centrifuged for 20 min at 1000 x g; plasma was then removed and stored at -20°C until required.

### **2.11.2 PGF2 $\alpha$ -induced luteolysis**

To synchronise follicular development and the stage of the ovarian cycle in the marmoset monkey luteolysis was induced in all control and experimental animals by

administering 1µg of a prostaglandin  $\text{PGF}_{2\alpha}$  analogue (cloprostenol, Planate, Coopers Animal Health Ltd., Crewe, UK) (Gilchrist et al., 2001). It has been shown that a single intramuscular injection of a  $\text{PGF}_{2\alpha}$  analogue induces luteolysis when given after day 8 of the luteal phase (Summers et al., 1985). Therefore the  $\text{PGF}_{2\alpha}$  analogue was injected intramuscularly between days 12-16 of the luteal phase, in the pre-treatment cycle, and this was designated day zero of the follicular phase. It has previously been shown that after luteolysis is induced by administration of  $\text{PGF}_{2\alpha}$  it takes approximately 10 days for dominant follicles to develop to the preovulatory stage (Summers et al., 1985). In addition,  $\text{PGF}_{2\alpha}$  administration is a commonly used tool in the investigation of marmoset luteal regression (Summers et al., 1985, Webley et al., 1989, Michael and Webley, 1993, Duncan et al., 1998, Fraser et al., 1999).

### **2.11.3 Treatment regimes**

Experiments were carried out in accordance with the Animals (Scientific Procedures) Act, 1986, and approved by the Local Ethical Review Process Committee. Administration of treatments was undertaken by staff at the primate centre under the supervision of Prof. Hamish Fraser. All experiments were carried out in accordance with the Animals (Scientific Procedures) Act, 1986. To synchronise the timing of the initiation of follicular development all control and experimental animals were given 1µg  $\text{PGF}_{2\alpha}$  analogue as described above. Specific details of the dose, timing and duration of treatment can be found in the relevant experimental chapter (Chapter 5). One of the parameters used to measure angiogenesis was cell proliferation. In order to visualise proliferation bromodeoxyuridine (BrdU; Boehringer Mannheim, Sussex, UK) was administered to all animals. BrdU is a thymidine derivative which is

incorporated into the DNA of proliferating cells during the S phase of the cell cycle.

Twenty milligrams, dissolved in 500µl physiological saline, was administered to all animals 1h prior to tissue collection.

#### **2.11.4 Collection of tissue**

Animals were sedated with 200µl ketamine hydrochloride (Park-Davis Veterinary, Pontypool, UK) intramuscularly, euthanised with a 400µl intravenous injection of Euthetal (sodium pentobarbitone, Rhone Merieux, Harlow, Essex, UK) and exsanguinated with a needle and syringe. Ovaries were removed immediately, weighed, and fixed in 4% NBF for 24h before paraffin wax embedding.

#### **2.11.5 Progesterone radioimmunoassay**

To monitor the marmoset ovulatory cycles plasma concentration was measured three times weekly for several cycles prior to use of the animal. Progesterone concentrations were also measured during, and post-, treatment. Progesterone radioimmunoassay was carried out by the in-house assay lab according to Smith et al., (Smith et al., 1990).

#### **2.11.6 ABT-898 ELISA**

The ABT-898 assay was carried out by the in-house assay lab. ABT-898 was measured by an ELISA, using biotinylated ABT-898 to capture and an antibody to ABT-898 as the reporter. Serum samples were diluted in assay buffer and run against standards also prepared in assay buffer. Each dilution level was assayed, and those that read on the linear part of the standard curve, in which the samples ran parallel to



that of the reference standard, were selected for analysis. If in the initial assay, values were below the limit of detection, samples were re-assayed neat and the standards spiked with an equivalent volume of mouse serum. Assay sensitivity was 2ng/well, and interassay variation based on low-, medium-, and high-quality controls were less than 10%. Concentration vs. time curves were constructed from ELISA-generated ABT-898 values obtained from individual animals. The pharmacokinetic parameter estimates were determined by fitting the serum concentration vs. time profile to a non-compartmental model (WinNonLin, version 2.0, Pharsight Corp., Mountain View, CA).

### **2.11.7 Specific immunohistochemistry techniques in the marmoset**

#### **2.11.7.1 Caspase-3 (*apoptotic cells*)**

Detection of the expression of activated caspase-3 in the marmoset was performed as described previously in 2.7.4.1.

#### **2.11.7.2 Bromodeoxyuridine (BrdU) (*proliferating cells*)**

5-bromo-2-deoxyuridine (BrdU) is a base analogue of thymidine, one of the bases of the nucleic acid found in DNA. Since the 1970s BrdU incorporation has been used as a tool for measuring DNA synthesis in cells and tissues and is a common chemical used in the detection of proliferating cells (Dolbeare, 1995). BrdU injected into animals one hour prior to euthanasia will incorporate into the DNA of cells replicating during the interval before euthanasia. Newly synthesised DNA will (partly) incorporate BrdU instead of thymidine and this can be visualised by

immunohistochemistry. Other endogenous markers of cellular proliferation are available, such as proliferating cell nuclear antigen (PCNA) and Ki67, although these are often thought of as 'soft' markers, as their expression is rarely restricted to a specific cell cycle phase, owing to time delays in synthesis and degradation (Rew and Wilson, 2000).

After performing antigen retrieval, the slides were washed three times with TBS for 5min and then blocked in NRS (diluted 1:5 in TBS containing 5% BSA) for 30min. The sections were incubated overnight at 4°C with an antibody to BrdU (Boehringer Mannheim 1170376) diluted 1:30 in NRS. After three 5min washes with TBS, a rabbit anti-mouse secondary antibody was added 1:60 in NRS and incubated for 40min, followed by mouse APAAP 1:100 in NRS for 40min. Slides were washed with TBS and then transferred to NBT buffer (40ml 0.5M MgCl<sub>2</sub>, 40ml 1M Tris/1M NaCl, 320ml H<sub>2</sub>O) for 10min before staining with NBT solution (10ml NBT buffer + 45µl NBT substrate (Boehringer Mannheim) + 35µl Xphosphate (Boehringer Mannheim) + 10µl levamisole) for 10-15min. Reactions were stopped in tap water, slides were then counterstained in haematoxylin for approximately 10 seconds before being air dried, cleared in xylene and mounted using Pertex.

#### **2.11.7.3 CD31 (endothelial cells)**

After performing antigen retrieval, the slides were washed three times with TBS for 5min and then blocked in NRS for 30min. The sections were incubated overnight at 4°C with an antibody to CD31 antibody (Dako M0823) diluted 1:20 in NRS. After three 5min washes with TBS, a rabbit anti-mouse secondary antibody was added

1:60 in NRS and incubated for 40min, followed by mouse APAAP 1:100 in NRS for 40min. Slides were washed with TBS and then staining was detected using fast red in solution at 1mg/ml in fast red buffer (20mg naphol AS-MX phosphate, 2ml dimethyl formamide to 98ml 0.1M tris, pH 8.2) and left for approximately 20-30min until a red colour developed. Reactions were stopped in tap water. Slides were air dried before being cleared in xylene and mounted in Pertex

#### **2.11.7.4 BrdU/ CD31 Co-localisation**

To allow the detection of two antigens on the same slide, two separate detection systems were used. For the purposes of this thesis, it was required that BrdU and CD31 were dual stained. The antibodies used were raised in different species, sheep and mouse respectively, ensuring that no cross reactivity occurs. The secondary antibody to be used for both antibodies was raised in rabbit. Thus the blocking serum is the same for both antibodies, in this case NRS.

After performing antigen retrieval, the slides were washed three times with TBS for 5min and then blocked in NRS for 30min. The sections were incubated overnight at 4°C with an antibody to CD31 diluted 1:20 in NRS. After three 5min washes with TBS, a rabbit anti-mouse secondary antibody was added 1:60 in NRS and incubated for 40min, followed by mouse APAAP 1:100 in NRS for 40min. Slides were washed with TBS and then staining was detected using fast red as per the CD31 protocol described above (2.11.7.3)

Sections were then washed and Sequenzas flushed through with TBS before performing a Streptavidin block for 15min, followed by TBS washes and a Biotin

block for 15min. Slides were washed with TBS before the sheep BrdU primary antibody (Fitzgerald Industries international, Concord, MA, USA) was added, diluted to 1:5000 in NRS and incubated overnight at 4°C. After three 5min washes with TBS, a rabbit anti-sheep biotinylated secondary antibody was added 1:500 in NRS and incubated for 40min, followed by TBS washes and then mouse ABC-AP (Dako K0376; 5ml Tris-buffer, 1 drop of bottle A and 1 drop bottle B) for 30min. Slides were then washed and transferred to NBT buffer for 10min before staining with NBT solution for 10-15min. Reactions were stopped in tap water, slides were then counterstained in haematoxylin for approximately 10 seconds before being air dried, cleared in xylene and mounted using Pertex.

#### **2.11.7.5 Tie-2**

Detection of the expression of Tie-2 in the marmoset was performed as described previously in 2.7.4.2.

### **2.11.8 Image Analysis**

#### **2.11.8.1 Caspase-3**

The total area of caspase-3 staining in the granulosa cell layer of each follicle was determined using Image Pro Plus software (Media Cybernetics). For each follicle, the section containing the oocyte with a nucleus present was used for quantification of staining. The cross-sectional area of staining for activated caspase-3 was calculated as a proportion of total cross-sectional area of granulosa cells within each follicle.

**2.11.8.2 Tie-2**

The expression of Tie-2 was analysed manually at x40 magnification using a tally counter. Expression was determined in the granulosa and thecal layers of preantral, early antral and mid-antral follicles with the intensity of Tie-2 expression being classified in each follicle using a visual scoring system (1=weak; 2=moderate; 3=strong staining). The percentage of follicles at each intensity was then calculated for each follicle stage and expressed as a mean HSCORE value (Budwit-Novotny et al., 1986). HSCORE is a semiquantitative analysis that has been shown to have low intra- and inter-observer error (Lessey et al., 2000). Only those follicles with a visible oocyte containing a nucleus were considered to ensure proper follicular classification.

**2.11.8.3 BrdU**

Sections were analysed under x40 magnification and quantified using Image ProPlus software (Media Cybernetics). The image analysis software was set up to analyse the number of BrdU positive cells and the total number of cells in the outlined cellular compartment of interest. This was done for both the thecal layer and granulosa cells. The proliferation index is expressed as the percentage of cells proliferating from the total number of cells in that compartment. Calculated for the granulosa and thecal compartments separately, the proliferation index is expressed as a mean value for each follicular stage.

**2.11.8.4 CD31**

The endothelial cell area was measured at x40 magnification and quantified using Image ProPlus software (Media Cybernetics). The whole area of the thecal

compartment and the CD31 positive area were measured, and the CD31 positive area was calculated per unit area of the thecal compartment. It is expressed as a mean value for each follicular stage.

#### **2.11.8.5 *BrdU/CD31 Co-localisation***

Dual-stained follicles were analysed manually at x40 magnification using a tally counter. The number of dual-stained cells (both BrdU and CD31 positive cells) and the total number of proliferating cells (BrdU positive cells) were counted in the thecal compartment. The proportion of dual-stained cells of proliferating endothelial cells (dual-stained cells) is expressed as a percentage of the total number of proliferating cells. The mean value was calculated for the number of follicles assessed within each follicular class and per animal. Non-endothelial cell proliferation was calculated by multiplying the total thecal proliferation index (BrdU index) by the percentage of proliferation that was not endothelial (not dual stained, BrdU positive cells on a dual stained slide), per follicle class per animal.

## **2.12 Statistical analysis**

Data are presented as mean  $\pm$  SEM. Results were analysed statistically by means of a one-way ANOVA. Although an ANOVA was chosen for the majority of the results it would have been possible to perform multiple t-tests to compare the different experimental groups but this could introduce errors and an ANOVA is a more powerful technique in complex analyses and allows easier interpretation of the results. An ANOVA uses an F-ratio to determine the statistical significance of the results and if a significant F-value is returned this can be followed up with post-hoc

tests to assess which groups are different from each other. In this thesis a statistically significant ANOVA was followed by a Bonferroni post-hoc test using Graph Pad Prism 5 software (Graph Pad Software, Inc. San Diego, CA) to determine significance between all of the experimental groups. For the results on change in follicle size (Fig 3.1, Fig 3.3) the results were analysed using paired two-tailed t-tests as only two groups were compared at a time and the results for Fig 5.4, Fig 5.5. Fig 5.6 were analysed by using unpaired two-tailed t-tests as the ovaries either came from control or treated animals. For the progesterone results (Fig 5.3), the post-treatment period data were subjected to statistical analysis and this was based on the duration of the normal luteal phase. This was achieved by comparing the area under the curve for pre-treatment and post-treatment cycles. P values  $<0.05$  were accepted as statistically significant.

## **Chapter 3 Development of a Novel *in vitro* Angiogenesis Assay**



### 3 Chapter 3 Development of a Novel *in vitro* Angiogenesis Assay

#### 3.1 Introduction

Angiogenesis is the formation of new blood vessels from existing blood vessels. It is rare in the healthy adult, except in the female reproductive tract, where angiogenesis occurs in a cyclical manner. Angiogenesis plays an important role in many pathological conditions including cancer, coronary artery disease, polycystic ovary syndrome and endometriosis and is therefore an area of great interest and much research is carried out in this field.

There are many models available to investigate angiogenesis, including both *in vitro* and *in vivo* systems. *In vitro* systems commonly used to investigate angiogenesis include the rat aortic ring assay, the chick aortic arch assay, the mouse metatarsal assay and human umbilical vein endothelial cell (HUVEC) bead assays (Nehls and Drenckhahn, 1995a, Nehls and Drenckhahn, 1995b, Auerbach et al., 2000, Auerbach et al., 2003, Staton et al., 2004, Goodwin, 2007, Nakatsu and Hughes, 2008), and whilst all of these methods have enhanced the understanding of angiogenesis and of the mechanisms involved in it, they also have limitations.

Angiogenesis is primarily microvascular event so the use of large vessels, such as in the rat aortic ring assay, the chick aortic arch assay and HUVEC bead assays is not truly representative of *in vivo* angiogenesis. The use of tissues that have been obtained from growing embryos is also not representative of physiological

angiogenesis as the cells are undergoing rapid cell division and proliferation, before explantation. Therefore the vessel outgrowth seen is not representative of the stimulation of angiogenesis from non-proliferative endothelial cells (Staton et al., 2004, Goodwin, 2007). In addition, these assays are time consuming – often taking up to 2 weeks to provide results (Auerbach et al., 2000, Auerbach et al., 2003, Staton et al., 2004, Goodwin, 2007) and many are limited by variability of the tissues used, making it necessary to use large numbers to achieve significance.

There are also many *in vivo* angiogenesis models used in current research and these are more representative of the stimulation of angiogenesis under normal *in vivo* conditions. However, they are limited by numerous challenges due to the complexity of the system used and the need to reduce the use of live animals in scientific research.

Of the many factors involved in the regulation of angiogenesis, vascular endothelial growth factor (VEGF) is considered to be the key factor involved in stimulating angiogenesis. Various studies have highlighted the role of VEGF in ovarian physiology by demonstrating its essential role in follicular development, as well as its impact upon angiogenesis (Geva and Jaffe, 2000, Danforth et al., 2003, Zimmermann et al., 2003, Abramovich et al., 2006). In addition, the observation that VEGF is up-regulated in many pathological conditions (Krasnow et al., 1996, Agrawal et al., 1998b) has led to the proposed use of anti-VEGF treatments, such as Aflibercept (VEGF Trap), to inhibit pathological angiogenesis (Strawn et al., 1996, Presta et al., 1997, Taylor et al., 2007). Indeed, investigation regarding the use of

Aflibercept in cancer treatment is already underway (Ferrara et al., 2007). With these facts in mind, Aflibercept (VEGF Trap) presents an ideal ‘gold standard’ molecule to investigate *in vitro*.

Aflibercept is a soluble VEGF receptor fusion protein with anti-angiogenic actions mediated by the inhibition of the pro-angiogenic factor VEGF. This inhibition is achieved by direct binding to VEGF, rendering it unable to act via its receptors VEGFR-1 (KDR) and VEGFR-2 (Flt-1) (Fraser, 2006). These receptors mediate the pro-angiogenic effects of VEGF via actions on endothelial cell proliferation (Ferrara and Davis-Smyth, 1997, Pandya et al., 2006) and a possible role in immature vessel survival (Folkman, 2004). However, Aflibercept is a high molecular weight protein and it may be that its size restricts its accessibility *in vitro* and *in vivo* so for this reason the action of a small molecule tyrosine kinase inhibitors of VEGF, ZM323881 (Whittles et al., 2002) will also be examined. ZM323881 selectively inhibits VEGFR-2, a range of other tyrosine kinases. It also inhibits VEGF-A induced endothelial cell proliferation, *in vitro* (Whittles et al., 2002).

Overall these factors show that the development of a novel *in vitro* angiogenesis assay, that would be representative of *in vivo* angiogenesis, be easily reproducible and provide a method for mechanistic studies would be a valuable tool in angiogenesis research. As angiogenesis occurs in a cyclical manner in the female reproductive tract and as, to our knowledge, no system exists for the manipulation and assessment of follicular angiogenesis in an *in vitro* setting, it was suggested that the ovary would be an ideal tissue for investigation into angiogenesis. In fact, the

female reproductive system is unique as no other healthy adult tissues undergo spontaneous angiogenesis. The tissues in ovarian follicles recruit blood vessels from the ovarian stroma at the preantral stage of development, to form vascular sheaths in the thecal layer and, as this process occurs naturally, it was proposed that the angiogenic properties of ovarian follicles could be utilised to provide a novel angiogenesis assay. Therefore, in this chapter an *in vitro* angiogenesis assay has been developed and optimised using the culture of intact isolated pre- and early antral rat follicles, to provide a new approach to the study of follicular angiogenesis.

## 3.2 Materials and Methods

### 3.2.1 Follicle culture

For all experiments, preantral/early antral follicles were cultured individually in flat-bottomed 12-well plates, embedded in the centre of a 20µl droplet of growth-factor reduced Matrigel. The Matrigel provides a three-dimensional extracellular matrix support for follicle growth and allows the vascular outgrowths to develop in three-dimensions, as they would *in vivo*. Follicles were incubated for 6 days in a sterile humidified air atmosphere with 5% CO<sub>2</sub> at 37°C in 600µl serum-free medium, with culture medium refreshed every 48h.

To optimise this assay, a variety of media and media supplements were tested to investigate which were best for follicle health but also best for the development of angiogenic outgrowths.

In order to test the efficacy of the assay, the known angiogenesis inhibitor Aflibercept (Regeneron Pharmaceuticals, NY, USA) was used. Aflibercept is a soluble decoy receptor created by fusing the extracellular domains of the human VEGF receptors (VEGFR-1 and -2) to the Fc portion of a human immunoglobulin. The incorporation of the Fc domain results in homodimerisation of the recombinant protein, creating a high affinity Aflibercept (Holash et al., 2002). Follicles were randomly placed into groups of 6 and cultured in control medium or in the presence of 1, 10, or 100µg/ml Aflibercept. Medium containing the Fc portion of human immunoglobulin (Regeneron) at 100µg/ml was used as the control.

To investigate whether the small molecule ZM323881 (Tocris Bioscience, Bristol, UK) would have greater efficacy than the larger molecule Aflibercept follicles were randomly placed into groups of 12 and cultured in control medium or in the presence of 10, 100, or 1000nM ZM323881.

### **3.2.2 Immunohistochemistry**

To confirm the endothelial nature of the angiogenic sprouting, a sample of follicles was analysed for labelling for Dil-Ac-LDL, CD31,  $\alpha$ SMA and also dual stained for CD31 and  $\alpha$ SMA (see Chapter 2). Staining was detected using confocal microscopy at 488nm and 568nm.

### **3.2.3 Activated caspase-3 immunohistochemistry**

To determine the localization and changes in the number of dying cells, a rabbit antibody to activated caspase-3 was used on sections from fixed follicle slides. The sections were incubated overnight at 4°C with activated caspase-3 antibody (1:100 in NGS) and visualization was achieved by 3-3'-diaminobenzidine (DAB) Substrate (see Chapter 2).

### **3.2.4 Granulosa Cell Experiments**

#### ***3.2.4.1 Isolation and culture of granulosa cells***

To investigate whether Aflibercept can induce apoptosis in granulosa cells directly, isolated granulosa cells were cultured in 0.1ml M199 medium containing  $1 \times 10^4$  cells/well. Following overnight preincubation in a sterile humidified air atmosphere with 5 % CO<sub>2</sub> at 37°C, prewarmed 0.1ml medium containing with 0, 1, 10 or 100

µg/ml Aflibercept was added to each well for 48h. This potential effect was also investigated after treatment with 0, 10, 100 or 1000nM ZM323881. Treatments were carried out in triplicate and experiments were repeated three times.

#### **3.2.4.2 Caspase-3 and -7 activity assay**

To measure caspase-3 and -7 activities in isolated granulosa cells, the Caspase-Glo 3/7 assay was performed according to the manufacturer's instructions, 48h after treating the cells (see Chapter 2). Treatments were carried out in triplicate and experiments were repeated three times to reduce the possible effects of biological variability. As serum can generate a background caspase activity signal, an additional two wells contained cell culture medium and carrier solution without any cells and TNFα was used as a positive control.

### **3.2.5 Quantification**

#### **3.2.5.1 Analysis of Angiogenic Sprouting**

To assess the impact of the treatments on angiogenic sprouting, follicles were examined and photographed directly in the 12-well plates under an inverted light microscope, at x10 magnification, with Axiovision image capturing software on days 0 and 6 of the culture period. The total area of angiogenic sprouting for each follicle was determined using Image Pro Plus software, by drawing an area of interest around the outgrowths (but excluding the follicle) and the sum of the area of all sprouts was measured and calculated by the software. The mean area of angiogenic sprouting for each treatment was then compared to the control.

### **3.2.5.2 Analysis of immunohistochemistry**

The total area of caspase-3 staining in the granulosa cell layer of each follicle was determined using Image Pro Plus software. For each follicle, the section containing the oocyte with a nucleus present was used for quantification of staining. The cross-sectional area of staining for activated caspase-3 was calculated as a proportion of total cross-sectional area of granulosa cells within each follicle.

### **3.2.6 Statistical Analyses**

Data are presented as mean  $\pm$  SEM and all results were analysed statistically using Graph Pad Prism 5 software (Graph Pad Software). Figures 3.1 and 3.3 were analysed by paired two-tailed t-tests with a 95% confidence interval to compare the day 0 data with day 6 data and significance is indicated by \*. The other results were analysed by means of a One-way ANOVA with a Bonferroni post-hoc test performed if the 95% confidence interval was reached to determine significance between all of the data groups. P values  $<0.05$  were accepted as statistically significant.



### 3.3 Results

Throughout this study ovaries from the 21-day-old rats used for the angiogenesis assay were fixed, sectioned and stained for both haematoxylin and eosin and activated caspase-3. The ovaries contained large numbers of pre- and early antral follicles and very little staining for activated caspase-3 was visible.

#### 3.3.1 Optimisation of Medium

To develop and optimise an *in vitro* angiogenesis assay intact pre- and early antral rat follicles were isolated from 21-day-old Wistar rats and cultured for six days in various types of medium, containing a variety of supplements. Previous preliminary experiments (unpublished data) had been carried out and in these experiments follicles were cultured in endothelial basal medium (EBM)-2 supplemented with BSA, heparin and GA-1000 – the Basic control. Follicles cultured in this medium were not optimally healthy and had few angiogenic outgrowths, despite the system being intended to produce outgrowths.

To optimise this assay improvement in the health of the follicles and production of an increased number of angiogenic outgrowths was required. Although other types of media are usually used for follicle culture, EBM-2 medium has been shown to be important for the development of angiogenic outgrowths and is often used for human umbilical vein endothelial cell (HUVEC) network assays (Bishop et al., 1999, Albaugh et al., 2001, Kawai et al., 2007, Ye and Yuan, 2007). Therefore follicles were cultured in EBM-2 medium with various types of supplements to determine which would provide the best conditions for the assay.

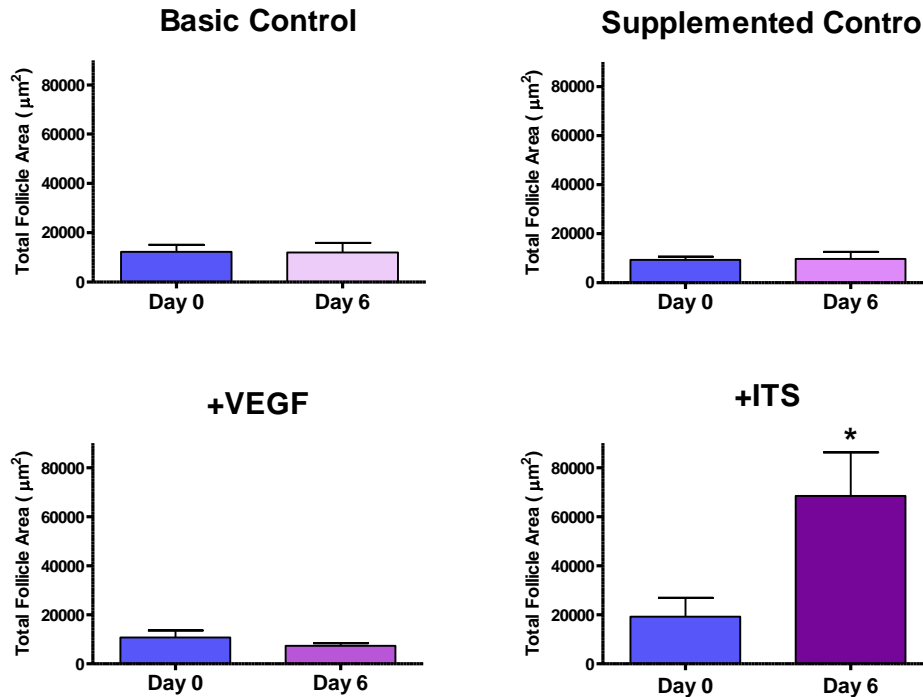
Follicles were cultured in the Basic control (n=12) (as described above), in EBM-2 medium supplemented with BSA (0.1%), L-glutamine (3mM), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), and L-ascorbic acid sodium salt (50 µg/ml) (Thomas 2001) – the Supplemented control (n=8). Follicles were also cultured in the supplemented control treated with insulin (10.0ng/ml), transferrin (2.5µg/ml) and selenium (4.0ng/ml) (ITS) (n=8) or VEGF (n=9) and then analysed for follicle health and development of angiogenic outgrowths. ITS was added to the culture medium as previous experiments have shown that these factors support follicle growth *in vitro* (Wright et al., 1999, Jeong et al., 2008). In addition, it has been used in *in vitro* maturation systems as it can improve oocyte health and growth (De La Fuente et al., 1999, Gardner et al., 2001, Herrick et al., 2004). We found that follicles cultured in the supplemented control treated with ITS were healthier than follicles cultured in other types of media and also produced more angiogenic outgrowths. Therefore it was decided that for future experiments, follicles would be cultured in EBM-2 medium supplemented with BSA (0.1%), L-glutamine (3mM), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), L-ascorbic acid sodium salt (50 µg/ml) and insulin (10.0ng/ml), transferrin (2.5µg/ml) and selenium (4.0ng/ml) (ITS).

As EBM-2 has been found to be important for the development of outgrowths but is not ideal for follicle health, different types of media that are commonly used for follicle culture were also investigated to establish whether it is possible for follicles to develop angiogenic outgrowths in these types of medium. Follicles were cultured in M199 medium, McCoy's medium, EBM2 treated with Activin A and McCoy's treated with Activin A, all treated with BSA (0.1%), L-glutamine (3mM), penicillin

(100 IU/ml), streptomycin (0.1 mg/ml), and L-ascorbic acid sodium salt (50 µg/ml) and ITS. Activin A was investigated at a concentration of 100ng/ml as it has been shown to improve follicle and oocyte health in cultures (Telfer et al., 2008).

### **3.3.2 Determination of follicle health**

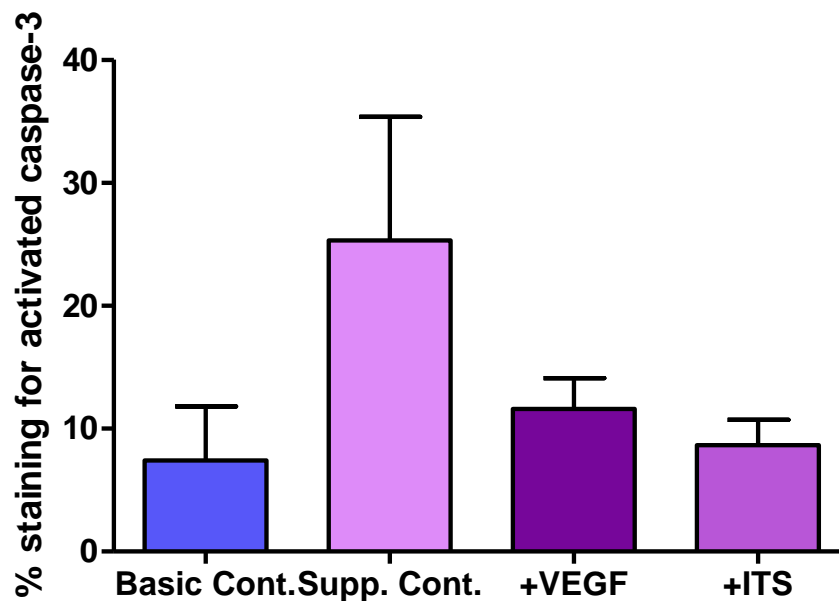
One of the indicators of follicle health in culture is an increase in size over the culture period and this was investigated by culturing follicles in EBM-2 medium with a variety of supplements to determine the optimum conditions for follicle health. Follicles were cultured for six-days and they were measured at the start and end of the culture period so that the change in follicle size could be monitored. Follicle size on day 0, for the different treatment groups, was analysed using a One-way ANOVA with a Bonferroni post-hoc test to determine any statistical difference in the initial follicle size and no difference was found (results not shown). Follicles cultured in the basic control, the supplemented control and the supplemented control treated with VEGF showed no significant increase in size. However, follicles cultured in the supplemented control treated with ITS showed a significant increase in follicle size ( $P<0.05$ ) over the six-day culture period (Figure 3.1).



**Figure 3.1 The change in follicle size over the 6-day culture period, for the different media treatments**

*Isolated preantral and early antral follicles were cultured in EBM2 media treated with a variety of supplements including vascular endothelial growth factor (VEGF) and insulin, transferrin and selenium (ITS) (n=6 per treatment) to determine optimum conditions for follicle culture. The size of the follicles before and after the culture was measured using Image Pro-Plus software as an indicator of follicle health. These data were analysed and compared using paired two-tailed t-tests to determine any significance between the day 0 and day 6 results. This showed that follicles cultured in the supplemented control +ITS significantly increased in size (\* $P < 0.05$ ) over the six days. Experiments were repeated in triplicate.*

To further investigate follicle health, follicles were fixed, sectioned and stained for activated caspase-3. Follicles cultured in the supplemented control treated with ITS had lower levels of staining for activated caspase-3 than follicles in the other groups, although levels of staining were similar to those seen in the basic control (Figure 3.2), further suggesting that the addition of ITS to the media improves the health of cultured follicles.

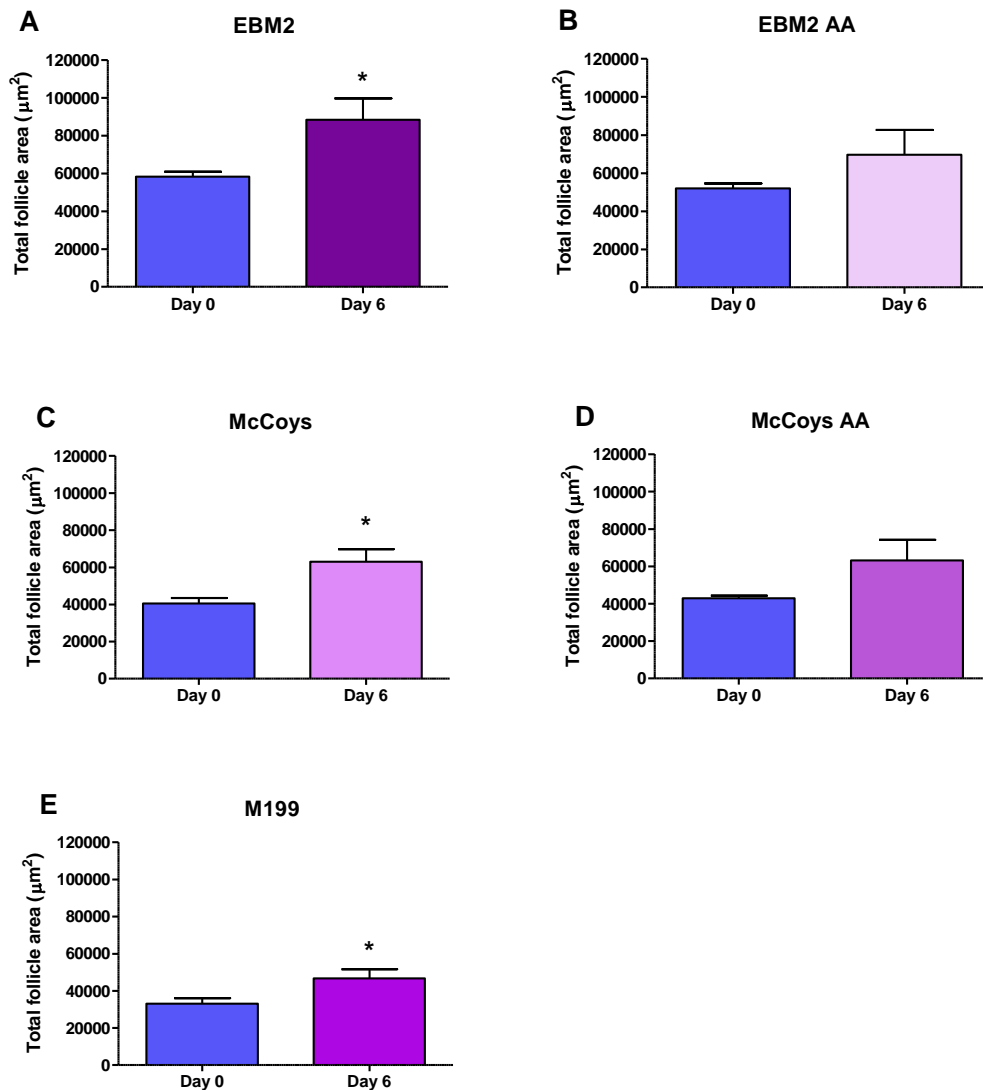


**Figure 3.2 The change in levels of staining for activated caspase-3 for the different media treatments**

*Isolated preantral and early antral follicles were cultured in EBM2 media treated with a variety of supplements including vascular endothelial growth factor (VEGF) and insulin, transferring and selenium (ITS) (n=6 per treatment) and at the end of culture these follicles were fixed, sectioned and stained for activated caspase-3 to determine the level of apoptosis in these follicles, as an indicator of follicle health. These data were analysed using a One-way ANOVA to compare all the different treatment groups to the Basic Control. A difference between the treatment groups was apparent but it was not statistically significant. Experiments were repeated in triplicate.*

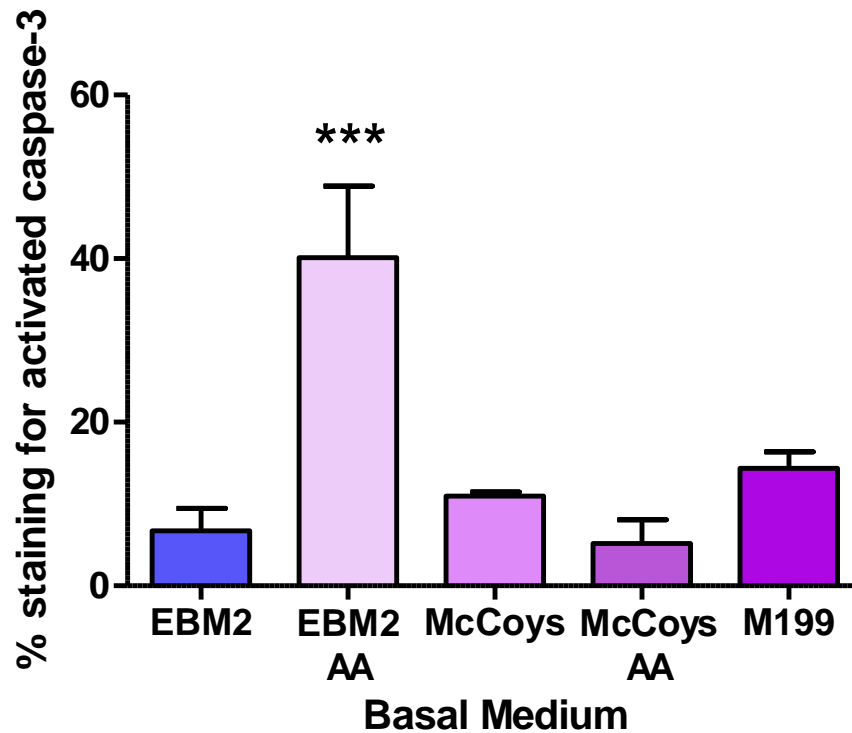
Different types of media have different compositions and are designed to support different cell types, therefore follicles were also cultured in various types of basal media to determine if a specific medium would be more beneficial for the health and growth of the isolated follicles. Follicle size on day 0, for the different treatment groups, was analysed using a One-way ANOVA with a Bonferroni post-hoc test to determine any statistical difference in the initial follicle size (results not shown) and there was a small difference in initial follicle size in the different groups. This difference in initial follicle size could be due to the follicles being at different stages

of development as both pre-antral and early antral follicles were isolated and pooled before being cultured. As both stages of follicles could have been used in this experiment this may have affected the final size of the different follicles, however, the follicle size on day 6 is directly compared to that of the same follicle on day 0 and it is the change in follicle size during culture, not the actual follicle size, which was used when comparing the different treatment groups. It could still have been possible that different sized follicles at the start would grow differently in the various media investigated, but as it was found that all follicles increased in size when cultured in specific media, it suggests that this did not affect the results or conclusions in any major way. Follicles cultured in McCoys medium, EBM2 medium and M199 medium showed a significant increase in follicle size over the culture period, whereas follicles cultured in McCoys medium with Activin A and EBM2 medium with Activin A showed no significant change in follicle size (Figure 3.3). In addition, these follicles were fixed, sectioned and stained for activated caspase-3 as an additional indicator of follicle health (Figure 3.4)



**Figure 3.3 The change in follicle size over the 6-day culture period, for the different media types**

Isolated preantral and early antral follicles were cultured in a variety of media including traditional follicle culture media (McCoys and M199) as well as media used for vascular cell culture (EBM2) ( $n=6$  per treatment) to determine the optimum media for this assay. The size of the follicles before and after the culture was measured as an indicator of follicle health. These data were analysed and compared using paired two-tailed  $t$ -tests to determine any significance between the day 0 and day 6 results. This showed that follicles cultured in EBM2 medium, McCoys medium and M199 medium significantly increased in size ( $*P<0.05$ ) over the six days. Experiments were repeated in triplicate.



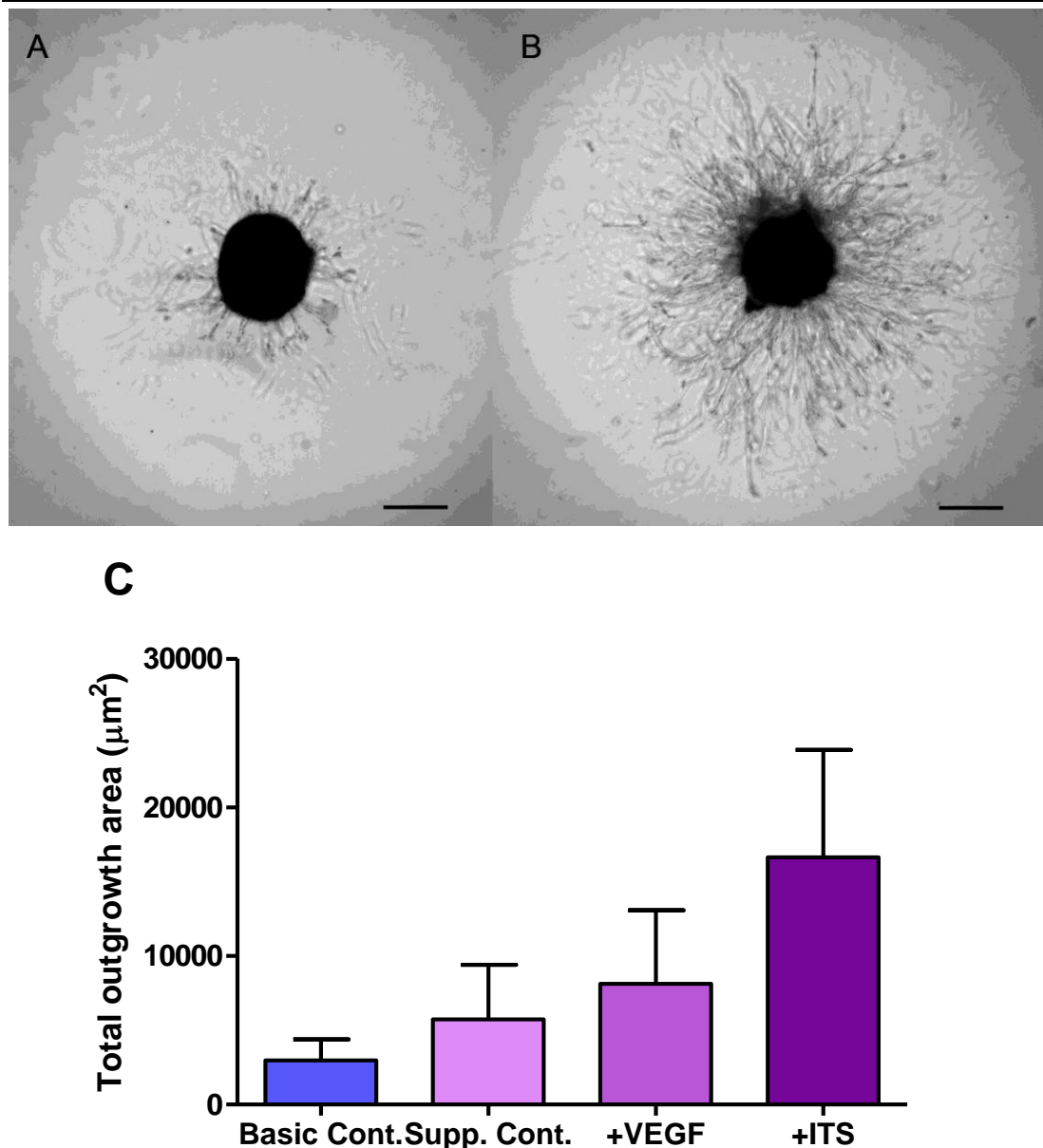
**Figure 3.4 The change in levels of staining for activated caspase-3 for the different media types**

Isolated preantral and early antral follicles were cultured in a variety of basal media including traditional follicle culture media (McCoys and M199) as well as media used for vascular cell culture (EBM2) ( $n=6$  per treatment) and at the end of culture these follicles were fixed, sectioned and stained for activated caspase-3 to determine the level of apoptosis in these follicles, as an indicator of follicle health. These results were analysed statistically using a One-way ANOVA with a Bonferroni post-hoc test to compare all treatment groups to EBM2. Follicles cultured in EBM2 medium, McCoys medium, McCoys medium with Activin A (McCoys AA) and M199 medium had low levels of staining for activated caspase-3, indicating that follicles cultured in these types of media were healthy. Follicles cultured in EBM2 medium with Activin A (EBM2 AA) had significantly ( $***P<0.001$ ) higher levels of staining for activated caspase-3 than the EBM2 cultured follicles, suggesting that these follicles were atretic. Experiments were repeated in triplicate.



### **3.3.3 Establishment of angiogenesis**

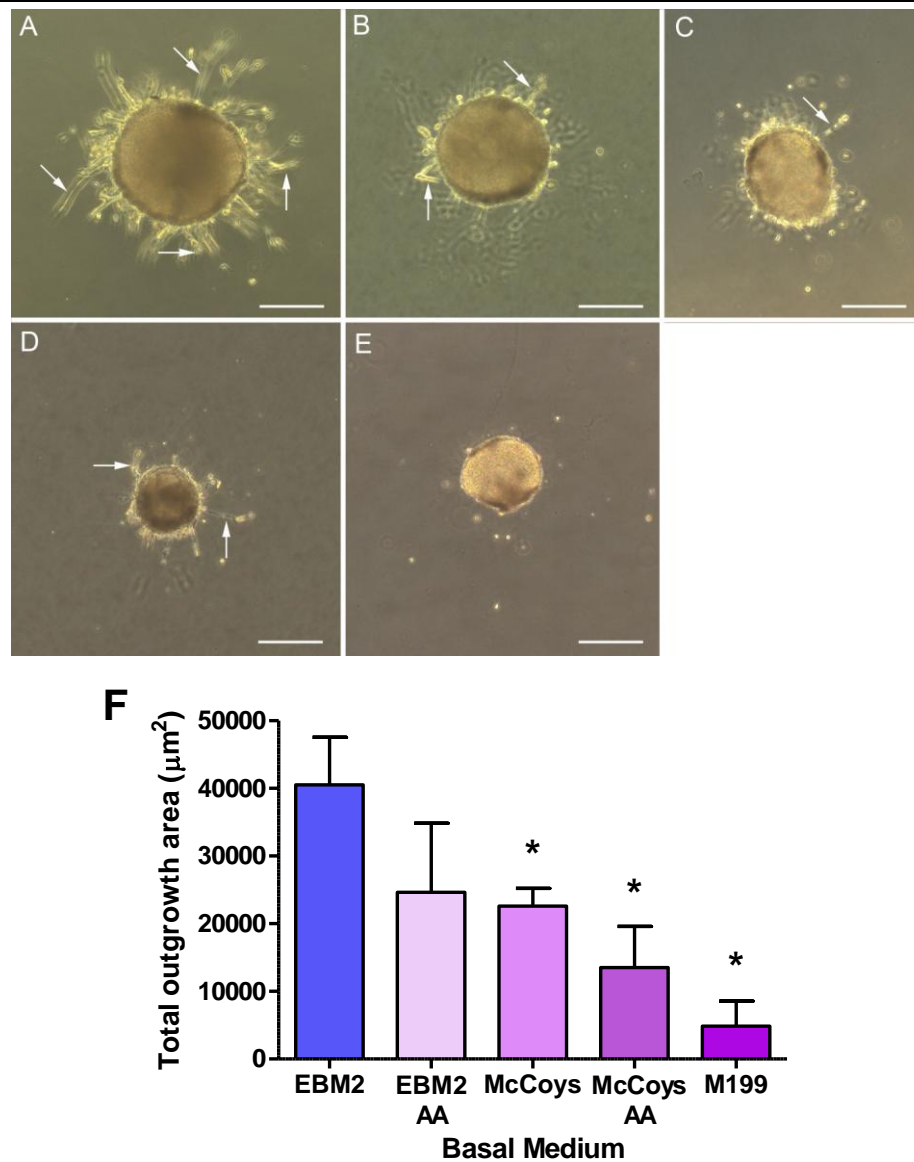
These studies showed for the first time that follicles in culture develop angiogenic outgrowths and that follicles cultured in the supplemented control treated with ITS produced more angiogenic outgrowths compared to follicles cultured in the basic control, the supplemented control and in the supplemented control treated with VEGF (Figure 3.5). Due to the variability between follicles this result was not significant but a clear trend was observed. It was anticipated that these vessels would grow from the cultured follicles as the thecal layer is already vascularised in the stages of follicles cultured in this assay and therefore blood vessels would be present in the thecal layer of these isolated follicles.



**Figure 3.5** The change in angiogenic outgrowth area over the 6-day culture period, for the different media treatments

Isolated preantral and early antral follicles were cultured in EBM2 media treated with a variety of supplements including vascular endothelial growth factor (VEGF) and insulin, transferring and selenium (ITS) ( $n=6$  per treatment) to determine optimum conditions for follicle culture. At the end of the culture period the area of the angiogenic outgrowths was measured using Image Pro-Plus software. Representative images show follicles cultured in the basic control (A) and the supplemented control treated with ITS (B). The results were quantified (C) and analysed statistically using a One-way ANOVA to compare the differences between the different media treatments and the Basic Control. Follicles cultured with ITS were observed to have a higher area of angiogenic outgrowths but due to variability of size between the follicles the results were not significant, although a clear trend can be seen. Scale bars represent  $100\mu\text{m}$ . Experiments were repeated in triplicate.

EBM2 medium has been found to be important for the development of angiogenic outgrowths but is not ideal for follicle health. The question of whether other types of media would allow the development of outgrowths was therefore investigated. The different types of basal media have different nutritional composition and therefore some types of media are more suitable for certain cell types than others. Follicles cultured in EBM2 medium produced significantly more angiogenic outgrowths than follicles cultured in McCoys medium, McCoys medium with Activin A and in M199 medium and a clear trend in decreasing angiogenesis can also be seen for follicles cultured in the other types of media (Figure 3.6).



**Figure 3.6 The change in angiogenic outgrowth area over the 6-day culture period, for the different media types**

Isolated preantral and early antral follicles were cultured in a variety of media including traditional follicle culture media ((McCoys and M199) as well as media used for vascular cell culture (EBM2) ( $n=6$  per treatment) to determine the optimum media for this assay. At the end of the culture period the area of the angiogenic outgrowths was measured using Image Pro-Plus software. Representative images show follicles cultured in EBM2 medium (A), EBM2 medium plus Activin A (EBM2 AA) (B), McCoys medium (C), McCoys medium plus Activin A (McCoys AA) (D) and M199 medium (E). The results were analysed statistically using a One-way ANOVA with a Bonferroni post-hoc test to compare the differences between the treated follicles and the EBM2-cultured follicles (F). Follicles cultured in EBM2 medium produced significantly ( $*P < 0.05$ ) more angiogenic outgrowths than did follicles cultured in McCoys medium, McCoys AA or M199 medium. White arrows indicate the endothelial outgrowths. Scale bars represent  $200\mu\text{m}$ . Experiments were repeated in triplicate.

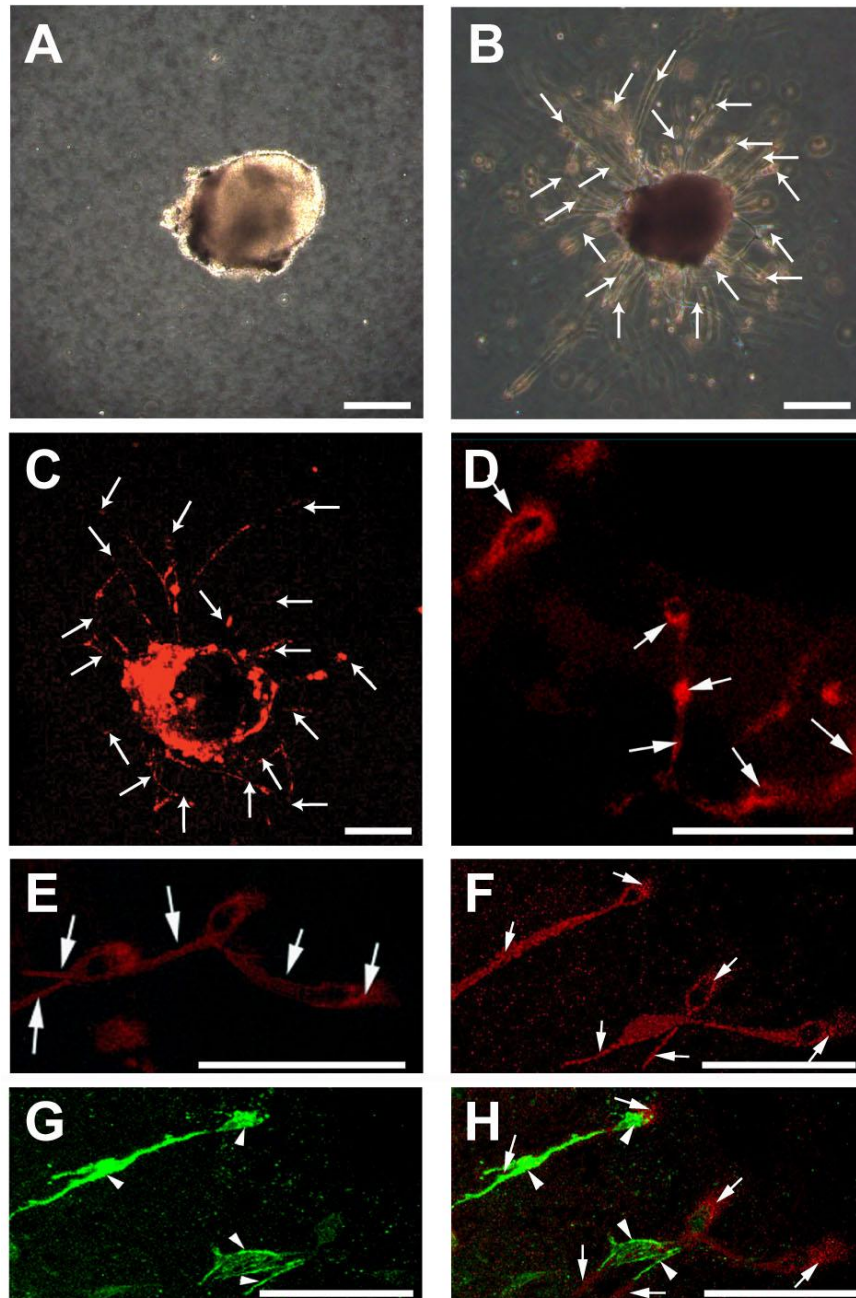
From these results it was decided that follicles cultured in EBM2 medium supplemented with BSA (0.1%), L-glutamine (3mM), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), and L-ascorbic acid sodium salt (50 µg/ml) and ITS produced significantly more angiogenic outgrowths than follicles cultured in a variety of other types of media and were as healthy as follicles cultured in McCoys medium, which is routinely used for follicle culture, so it was decided that this would be the optimal medium to use for the *in vitro* angiogenesis assay.

### **3.3.4 Characterisation of vascular outgrowths**

To establish whether angiogenesis occurred in our novel *in vitro* system, follicles were cultured for 6 days in serum-free EBM-2 medium. Follicles were photographed at the start and end of the culture period and the day 6 images were compared to the day 0 images. In Fig 1, representative images of a late preantral/early antral follicle at day 0 (Figure 3.7A) and day 6 (Figure 3.7B) of the culture period are shown. Follicles displayed angiogenic sprouting during the culture period (Figure 3.7B). Once it had been established that angiogenesis was occurring in the *in vitro* system, follicles were treated with endothelial cell markers to confirm that the cells were endothelial in nature. First, follicles were treated with the fluorescent endothelial cell marker Dil-Ac-LDL (20µg/ml, Serotec, Oxford, UK) (Voyta et al., 1984) on day 4 and the results were visualized on day 6, using fluorescence microscopy. Dil-Ac-LDL is a fluorescent-labelled acetylated low-density lipoprotein that is taken up via the “scavenger” metabolic pathway and is also be known as Dii-Ac-LDL. Dil-Ac-LDL is a commonly used and widely accepted method for the detection of endothelial cells (Voyta et al., 1984, Jimenez et al., 2000, Nor et al., 2000, Tong et

al., 2006, Werner et al., 2008, Burciaga-Nava et al., 2009, Janic et al., 2010, Szoke et al., 2011) and it has several advantages over labelling with other endothelial cell markers. Once the Dil-Ac-LDL is degraded by the lysosomal enzymes the fluorescent label, Dil, is internalized and accumulates in the intracellular membranes rendering them fluorescent. Scavenger receptors are a specific property of endothelial cells but not an exclusive one as they are also on macrophages; despite this Dil-Ac-LDL is one of the most specific endothelial cell markers available (Okaji et al., 2004). In our system Dil-Ac-LDL should specifically stain endothelial cells as macrophages which are not present in our system. Dil-Ac-LDL is able to identify endothelial cells from complex cell cultures as the most common contaminants in endothelial cell cultures (such as fibroblasts, smooth muscle cells, pericytes and epithelial cells) are not labelled by it.

In addition, control follicles were fixed at the end of culture and were stained for CD31, CD34 or  $\alpha$ SMA to further identify the nature of the outgrowths. Dil-Ac-LDL was incorporated into the outgrowths (Figure 3.7C), and the vessels were stained for CD31 (Figure 3.7D and F), CD34 (Figure 3.7E)  $\alpha$ SMA (Figure 3.7G) or CD31 and  $\alpha$ SMA (Figure 3.7H), showing that the outgrowths are endothelial in nature and representative of physiological angiogenesis.



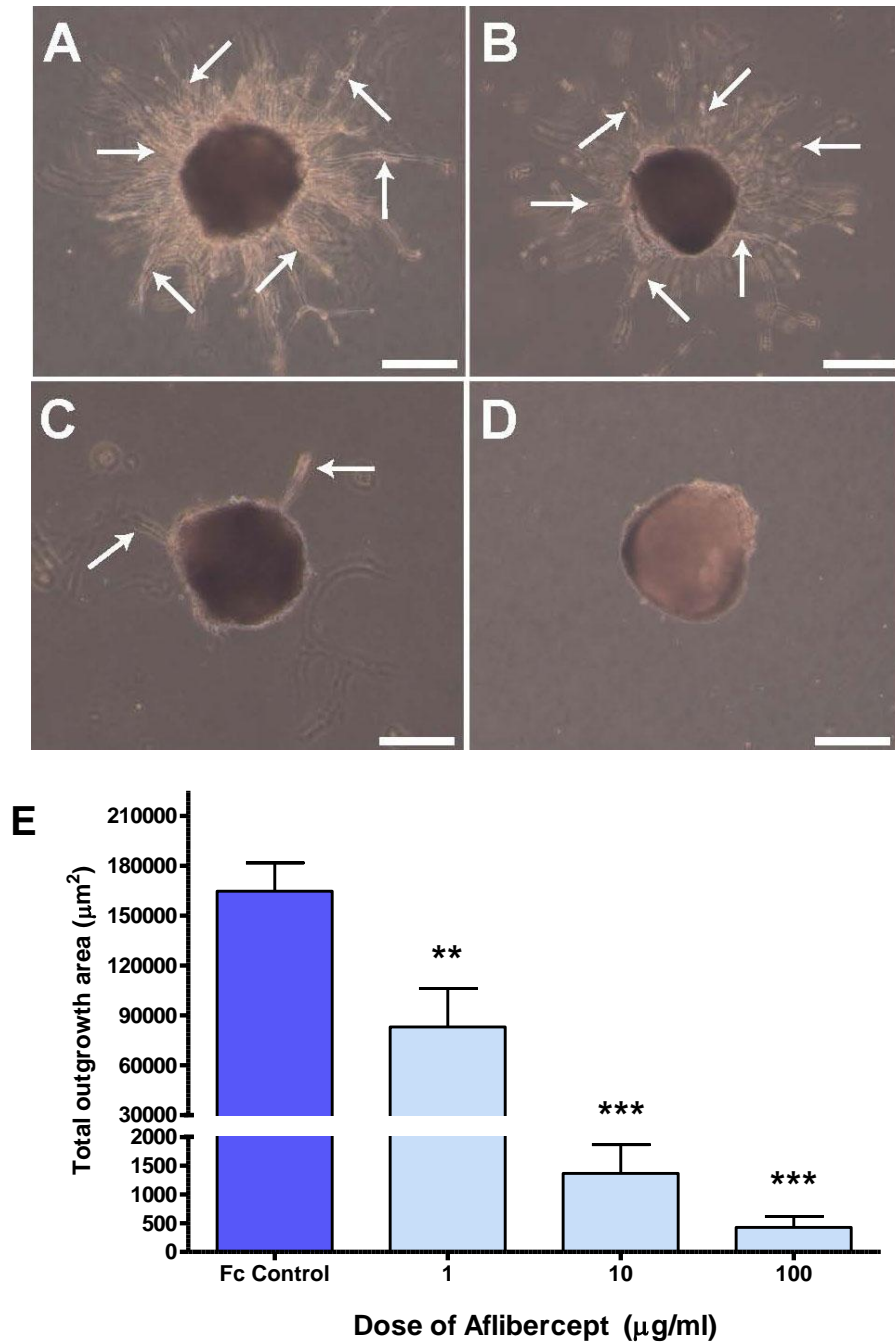
**Figure 3.7 Establishment of angiogenesis *in vitro***

Isolated preantral and early antral follicles were cultured and then fixed and stained by wholemount IHC to determine the cellular nature of the outgrowths. Representative images show a late preantral/early antral follicle on the first day of culture (A) and a follicle at the end of culture with vascular outgrowths (B). Vascular outgrowths were characterized by uptake of Dil-Ac-LDL (C). In addition, representative images of the outgrowths at higher magnification are shown labelled with CD31 (D) and CD34 (E). Dual labelling of outgrowths with CD31 and  $\alpha$ SMA was also performed, and the dual fluorescence image has been separated into the individual channels to show CD31 (F) and  $\alpha$ SMA (G) labelling. The CD31 labelling (red) is overlaid by the  $\alpha$ SMA labelling (green) in (H). Arrows indicate the endothelial outgrowths and labelling for CD31, CD34 and Dil-Ac-LDL. Arrowheads indicate labelling with  $\alpha$ SMA. Scale bars in A-C represent 200 $\mu$ m and in D-H represent 100 $\mu$ m.

### **3.3.5 Effect of Aflibercept on angiogenesis**

Once the medium had been optimised and the endothelial nature of the vessels confirmed, this culture system was employed to investigate the effect of various pro- and anti-angiogenic compounds. As Aflibercept is an established angiogenesis inhibitor it was used to establish the efficacy of this culture system and it was expected that Aflibercept would inhibit follicular angiogenesis. Preantral and early antral follicles (n=6 per treatment) were cultured in serum-free EBM2 medium (as described above) treated with control medium containing 100µg/ml Fc or with 1, 10 or 100µg/ml Aflibercept for 6 days. This resulted in a dramatic and significant reduction in follicular angiogenesis, in a dose dependent manner (Figure 3.8) (P<0.01).



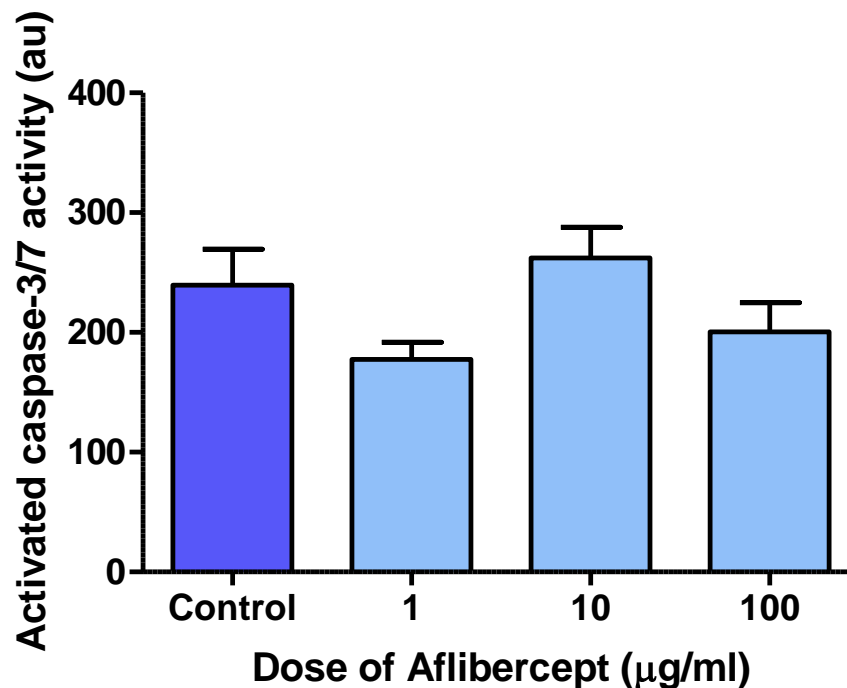


**Figure 3.8 Effect of Aflibercept on angiogenesis**

To determine the effect of Aflibercept on angiogenesis in this assay, isolated preantral and early antral follicles were cultured with varying doses of Aflibercept ( $n=6$  per treatment). At the end of the culture period the area of the angiogenic outgrowths was measured using Image Pro-Plus software. Representative images show a control follicle (A) and follicles after treatment with Aflibercept at 1 μg/ml (B), 10 μg/ml (C), or 100 μg/ml (D). Results were analysed statistically using a One-way ANOVA with a Bonferroni post-hoc test to compare the Aflibercept treated follicles to the control follicles. Treatment with Aflibercept resulted in a significant dose-dependent decrease in outgrowth area (E). \*\* indicates  $P<0.01$ , \*\*\* indicates  $P<0.001$ , in comparison with Fc Control. White arrows indicate the endothelial outgrowths. Scale bars represent 200 μm. Experiments were repeated in triplicate.

### 3.3.6 Effect of Aflibercept on follicular apoptosis

In addition, the effect of Aflibercept on follicular apoptosis was investigated to establish whether it affects follicle health as well as inhibiting follicular angiogenesis. This was investigated by culturing isolated granulosa cells with 0, 1, 10 or 100  $\mu\text{g/ml}$  Aflibercept for 48h followed by quantification of apoptosis using a luminescent caspase-3/7 assay (see Chapter 2). There was no difference in levels of activated caspase-3/7 activity in granulosa cells treated with increasing doses of Aflibercept (Figure 3.9), showing that Aflibercept does not induce apoptosis.



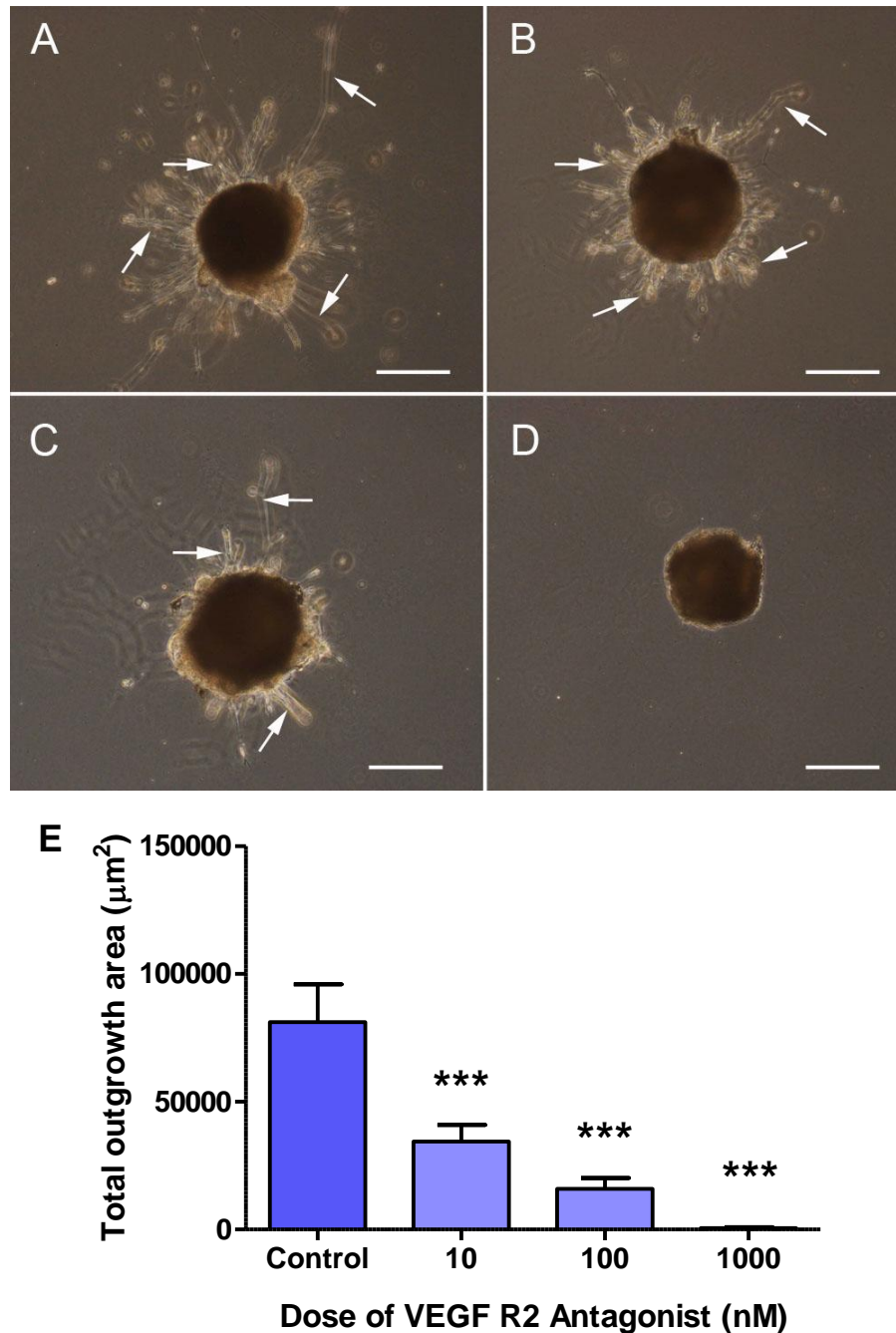
**Figure 3.9 Effect of Aflibercept on granulosa cell apoptosis**

*To determine the effect of Aflibercept on apoptosis in this assay granulosa cells were isolated from preantral and early antral follicles and they were cultured with varying doses of Aflibercept ( $n=6$  per treatment). At the end of the culture period these granulosa cells were analysed for expression of activated caspase-3/7 activity using a luminescent caspase activity assay, as an indicator of follicle health. A One-way ANOVA was performed on the results to determine any significance between the Aflibercept treated follicles and the control follicles and there was no significant difference in the levels of expression of activated caspase-3/7. Luminescence is given in arbitrary units (au). Experiments were repeated in triplicate.*

### **3.3.7 Effect of a VEGFR2 antagonist, ZM323881, on angiogenesis**

Aflibercept is a large molecule and it is conceivable that smaller molecules could have greater efficacy, being able to access tissues more readily than Aflibercept. A potent small molecule antagonist of the VEGFR2, ZM323881, was selected to investigate its effects on angiogenesis.

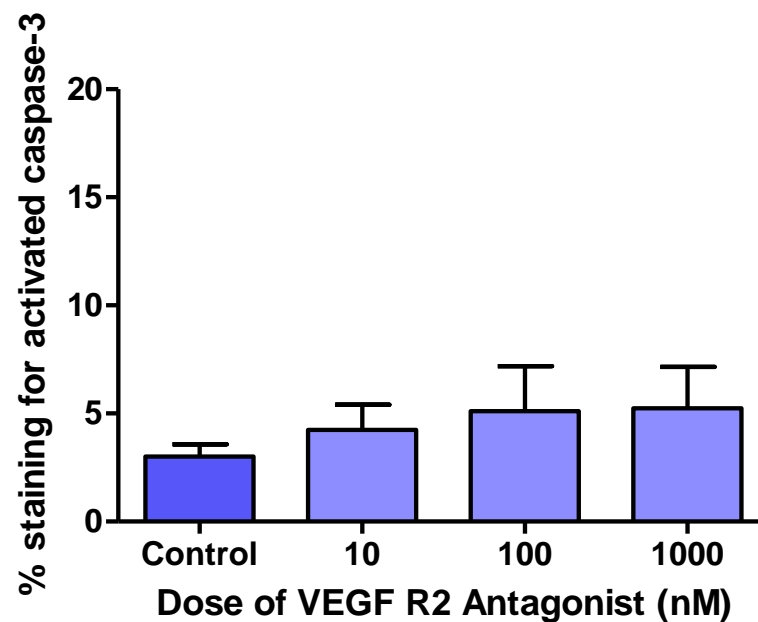
Preantral and early antral follicles (n=12 per treatment) were cultured in serum-free EBM2 medium treated with 0, 10, 100, or 1000nM ZM323881 for 6 days. This resulted in a significant decrease in follicular angiogenesis in a dose dependent manner (Figure 3.10) ( $P<0.001$ ).



### Figure 3.10 Effect of ZM323881 on angiogenesis

To determine the effect of ZM323881, a VEGFR2 antagonist, on angiogenesis, isolated preantral and early antral follicles were cultured with varying doses of ZM323881 ( $n=12$  per treatment). At the end of the culture period the area of the angiogenic outgrowths was measured using Image Pro-Plus software. Representative images show a control follicle (A) and follicles after treatment with ZM323881 at 10 (B), 100 (C), or 1000nM (D). The results were analysed statistically using a One-way ANOVA with a Bonferroni post-hoc test to compare the ZM323881 treated follicles to the control follicles. Treatment with ZM323881 resulted in a significant dose-dependent decrease in outgrowth area (E). \*\*\* indicates  $P<0.001$ , in comparison with the Control. White arrows indicate the endothelial outgrowths. Scale bars represent 200μm. Experiments were repeated in triplicate.

In addition, the follicles were fixed, sectioned and stained for caspase-3 to investigate the effect on follicle health and the results show that there was no difference in staining for activated caspase-3 with the different doses of ZM323881 (Figure 3.11).



**Figure 3.11** The change in levels of staining for activated caspase-3 for the different doses of ZM323881

To determine the effect of ZM323881 on apoptosis in this assay granulosa cells were isolated from preantral and early antral follicles and they were cultured with varying doses of ZM323881 ( $n=12$  per treatment). At the end of the culture period these granulosa cells were analysed for expression of activated caspase-3/7 activity using a luminescent caspase activity assay, as an indicator of follicle health. A One-way ANOVA was performed on the results to compare the ZM323881 treated follicles to the control follicles and treatment with varying doses of ZM323881 resulted in no change in the levels of expression of activated caspase-3. Luminescence is given in arbitrary units (au). Experiments were repeated in triplicate.

### 3.4 Discussion

A novel *in vitro* system has been developed that utilizes intact ovarian follicles to investigate follicular angiogenesis. This chapter has shown for the first time that follicles can develop angiogenic outgrowths in culture. In this system, tube-like structures with branch points representing angiogenic sprouting are formed and they readily take up endothelial cell markers.

Angiogenesis is a key feature of many pathological conditions and as anti-angiogenic therapies could become a powerful tool in the treatment of those conditions an *in vitro* system that allows easy quantification of the potential effect of these therapies, as well as being robust and reproducible, would provide a new approach to angiogenesis research. Although there are many models available for the investigation of angiogenesis they all have limitations. Moreover, our group studies the regulation of follicular angiogenesis so the availability of an *in vitro* follicular angiogenesis assay would enable manipulation of the process and of factors of interest.

In this chapter an *in vitro* assay has been developed and by culturing follicles in a variety of types of media and with various media supplements to establish the optimal culture conditions for follicle health and the development of angiogenic outgrowths. Different types of media have different compositions and this provides the different conditions that different cell types require to grow. Media commonly used for follicle culture, such as McCoys media, is composed of amino acids, vitamins, inorganic salts and serum and/or glucose which provide the nutrients

required for follicles to grow and develop *in vitro*. EBM-2 is specifically designed to support the growth of primary derived endothelial cells and contains no growth factors, cytokines or supplements and has a low serum content. The exact composition of EBM-2 is unknown but it appears to contain nutrients that stimulate angiogenesis in cultured endothelial cells. This property of EBM-2 is exploited in our assay and the growth of follicles in this media is enhanced by the addition of supplements to this basal medium. The cultured follicles were analysed for indicators of follicle health, including follicle growth and staining for activated caspase-3, and the area of angiogenic outgrowths was quantified. Follicles cultured in serum-free EBM2 medium with BSA (0.1%), L-glutamine (3mM), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), L-ascorbic acid sodium salt (50 µg/ml) and ITS produced more angiogenic outgrowths and were healthy when compared to follicles cultured in other media types or in EBM2 medium with different supplements. This suggests that, although EBM-2 is not typically used for follicle culture, that this medium would provide the best conditions to maintain follicle health whilst allowing angiogenic outgrowths to develop. Therefore it was decided that this was the optimal media to use for the *in vitro* angiogenesis assay.

As it has been shown for the first time that cultured follicles can produce angiogenic outgrowths, it was important to confirm that the outgrowths were endothelial in nature. The fluorescent marker Dil-Ac-LDL is commonly used to identify endothelial cells (Voyta et al., 1984) especially in angiogenesis assays (Gaffney et al., 1985, Yamazaki et al., 1995, Tong et al., 2006, Zan et al., 2008) and this chapter has shown that it is incorporated into the outgrowths from the follicles suggesting that they are

vascular in nature. To further clarify the vascular nature of the outgrowths control follicles were fixed and fluorescently labelled for CD31, CD34 and dual labelled with CD31 and  $\alpha$ SMA. The vessels labelled for the endothelial cell markers CD31 and CD34 and also dual labelled for CD31 and the pericyte marker  $\alpha$ SMA. When angiogenesis occurs *in vivo* pericytes surround the developing vessels and stabilise them, therefore the factors that the outgrowths produced in this assay label for show that they are endothelial in nature and are representative of physiological angiogenesis.

In addition to optimising this assay the efficacy of this assay was established. The extensive literature describing the anti-angiogenic effect of Aflibercept lead to the decision to use it as the 'gold standard' compound to determine the efficacy of this assay. Follicles cultured with increasing doses of Aflibercept resulted in a significant ( $P<0.01$ ) reduction in follicular angiogenesis, showing that this assay is a valid system for the investigation of follicular angiogenesis and also for the study of a variety of pro- and anti-angiogenic compounds.

As such the effect of the VEGFR2 antagonist ZM323881 on angiogenesis was also investigated and this was found to significantly inhibit follicular angiogenesis. The effect of this VEGF inhibitor was investigated as it was believed that it may have greater efficacy than Aflibercept, due to its smaller size. However, the inhibition of angiogenesis after treatment with ZM323881 was not as dramatic as that seen after treatment with Aflibercept suggesting that the size of Aflibercept does not impede its efficacy *in vitro*.



In summary, a novel *in vitro* assay for the quantification of follicular angiogenesis has been developed and optimised using the culture of isolated ovarian follicles. The evaluation of this culture system suggests that it is a valid method for the study of follicular angiogenesis and that the vessels produced are representative of *in vivo* angiogenesis. In addition, this system has been utilised to show that both Aflibercept and a VEGFR-2 antagonist are able to inhibit follicular angiogenesis, without inducing apoptosis. In the following chapter this system will be utilised to investigate the roles of factors involved in regulating *in vitro* follicular angiogenesis and their effects on follicle development and survival.

# **Chapter 4 Thrombospondin-1 Inhibits Follicular Angiogenesis and Promotes *Atresia in vitro***

## **4 Chapter 4 Thrombospondin-1 Inhibits Follicular Angiogenesis and Promotes Atresia *in vitro***

### **4.1 Introduction**

Follicles recruit blood vessels from the ovarian stroma to form vascular sheaths in the thecal layer at the preantral stage of development. These blood vessels surround the developing follicle and begin to circulate blood to and from the follicle, allowing nutrient and gaseous exchange (Geva and Jaffe, 2000). It is essential for rapidly expanding follicles to undergo increased angiogenesis, to sustain development of these follicles (Fraser, 2006).

Vascular endothelial growth factor (VEGF) is the key factor involved in promoting angiogenesis while thrombospondin-1 (TSP-1) is a putative anti-angiogenic factor (Ferrara, 2004, Tamanini and De Ambrogi, 2004, Greenaway et al., 2007). The members of the thrombospondin family TSP-1 and TSP-2 are large extracellular matrix glycoproteins and their effects are mediated through interaction with the cell surface receptors CD36 and CD47 (Dawson et al., 1997, Lawler, 2002). TSP-1 has been shown to inhibit angiogenesis both *in vitro* and *in vivo* and studies have shown that treatment with TSP-1 renders endothelial cells unable to respond to many pro-angiogenic factors (Lawler, 2002, Folkman, 2004). Several studies have used knock-out mice to show that the absence of TSP-1 leads to increased vascularisation (Lawler et al., 1998, Lawler et al., 2001, Wang et al., 2003, Sund et al., 2005, Greenaway et al., 2007), providing the first evidence for a role of TSP-1 *in vivo* as an inhibitor of angiogenesis. However, few studies have investigated the role of TSP-1

in the ovary. A previous study has made the interesting observation that TSP-1 mRNA and protein is up-regulated during follicular atresia *in vivo* (Thomas et al., 2008), suggesting that TSP-1 may be involved in the cessation of angiogenesis in follicles undergoing atresia. Alternatively, TSP-1 may be an autocrine/paracrine factor acting on granulosa and/or endothelial cells to promote follicular atresia, as the CD36 receptor for TSP-1 is present in both cell types (Thomas et al., 2008).

Studies in rat, bovine and marmoset models have demonstrated a decrease in both TSP-1 and CD36 expression as follicular development progresses (Greenaway 2005; Petrik 2002), leading to the suggestion that it inhibits angiogenesis during early follicular development. In addition, a correlation between down regulation of TSP-1 and tumour angiogenesis and invasiveness (Rodriguez-Manzanique et al., 2001, Miyanaga et al., 2002, Tanaka et al., 2002, Ren et al., 2006), has led to the proposed use of TSP-1 as a therapeutic inhibitor of angiogenesis (Weinstat-Saslow and Steeg, 1994, Streit et al., 1999, Greenaway et al., 2009). As angiogenesis is tightly regulated by both pro- and anti-angiogenic factors it is possible that these factors may be able to interact with each other. The direct effect of TSP-1 on granulosa cell apoptosis has not been investigated previously, nor has it been investigated in primary granulosa cells, thus one hypothesis of this chapter was to investigate whether TSP-1 is able to directly induce apoptosis in a more physiological setting. Phospho-ERK1/2 (pERK) and p38 mitogen-activated protein kinase (MAPK) are key factors involved in anti- and pro-apoptotic pathways, respectively, that either lead to the inhibition or induction of the caspase cascade (Donnini et al., 2004, Nakagawa et al., 2005, Ridnour et al., 2005). Previous studies have suggested that TSP-1 can interact with

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the ERK and MAPK pathways (Wilson et al., 1999, Sengupta et al., 2004, Tan et al., 2009) so the effect of TSP-1 treatment on the expression of pERK and p38 was investigated to determine the mechanisms of TSP-1-induced induction of apoptosis.

In this chapter, the role of TSP-1 in regulating *in vitro* follicular angiogenesis and its effect on follicle development and survival has been investigated, using the *in vitro* angiogenesis assay described in Chapter 3.

## 4.2 Materials and Methods

### 4.2.1 Follicle Culture

For all experiments, preantral/early antral follicles were cultured individually in flat-bottomed 12-well plates, embedded in the centre of a 20µl droplet of growth-factor reduced Matrigel. The Matrigel provides a three-dimensional extracellular matrix support for follicle growth and allows the vascular outgrowths to develop in three-dimensions, as they would *in vivo*. Follicles were incubated for 6 days in a sterile humidified air atmosphere with 5% CO<sub>2</sub> at 37°C in 600µl serum-free EBM-2 medium supplemented with BSA (0.1%), L-glutamine (3mM), penicillin (100 IU/ml), streptomycin (0.1mg/ml), transferrin (2.5µg/ml), selenium (4ng/ml), insulin (10ng/ml) and L-ascorbic acid, sodium salt (50µg/ml), with culture medium refreshed every 48h.

In order to test the effects of TSP-1, follicles were randomly placed into groups and cultured in control medium or in the presence of 1 (n=31), 10 (n=28), 100 (n=29) or 1000ng/ml (n=30) TSP-1 (EMP Genetech, Ingolstadt, Germany).

In order to test the effects of co-culturing TSP-1 with VEGF, follicles were randomly placed into groups and cultured in control medium (n=25) or in the presence of 10ng/ml TSP-1 with 0 (n=20), 1 (n=22), 10 (n=23), 100 (n=23) or 1000ng/ml (n=22) VEGF (Perbio Science UK Limited, Cramlington, UK).

#### **4.2.1.1 Analysis of Angiogenic Sprouting**

To assess the impact of the treatments on angiogenic sprouting, follicles were examined and photographed directly in the 12-well plates under an inverted light microscope, at x10 magnification, with Axiovision image capturing software on days 0 and 6 of the culture period. The total area of angiogenic sprouting for each follicle was determined using Image Pro Plus software. This was done by drawing an area of interest around the outgrowths (but excluding the follicle), and the sum of the area of all sprouts was calculated by the software. The mean area of angiogenic sprouting for each treatment was then compared to the control.

#### **4.2.2 Effect of TSP-1 on follicular atresia**

##### **4.2.2.1 Activated caspase-3 immunohistochemistry**

Follicles (n=21 per treatment group) were fixed at the end of the culture period in 4% NBF for 24h then stained with 70% ethanol containing eosin for 24h before being embedded in paraffin wax and serially sectioned. Tissue sections (5µm) were placed onto BDH SuperFrost slides. To determine the localization and changes in the number of dying cells, a rabbit antibody to activated caspase-3 was used on sections from fixed follicle slides. The sections were incubated overnight at 4°C with activated caspase-3 antibody (1:100 in NGS and visualization was achieved by 3-3'-diaminobenzidine (DAB) Substrate (see Chapter 2). For the negative controls, primary antibody was omitted.

**4.2.2.2 Analysis of immunohistochemistry**

The total area of caspase-3 staining in the granulosa cell layer of each follicle was determined (n=21 follicles per treatment) using Image Pro Plus software. For each follicle, the section containing the oocyte with a nucleus present was used for quantification of staining. The cross-sectional area of staining for activated caspase-3 was calculated as a proportion of total cross-sectional area of granulosa cells within each follicle.

**4.2.2.3 TdT-mediated dUTP-biotin nick end labelling (TUNEL)**

To provide an additional method for investigation of the effect of TSP-1 on cell death, whole follicles from control and TSP-1 treatment groups (n=3 per group) were subjected to TUNEL. Wholemount TUNEL was performed as described previously (Thomas et al., 2001). Follicles were visualized in PBS via fluorescence microscopy using a confocal microscopy at 488nm (green channel to show TUNEL labelled cells) and 568nm (red channel to show propidium iodide stained cells) simultaneously.

**4.2.3 Granulosa Cell Experiments**

To investigate whether TSP-1 can induce apoptosis in granulosa cells directly, isolated granulosa cells were cultured in 0.1ml M199 medium containing  $1 \times 10^5$  cells/ml. Following overnight preincubation in a sterile humidified air atmosphere with 5% CO<sub>2</sub> at 37°C, prewarmed 0.1ml M199 medium containing with 0, 10, 100 or 1000ng/ml TSP-1 was added to each well for 48h. Twenty-one-day old female Wistar rats were used as described previously (Chapter 2). To investigate the



possibility that TSP-1 induces apoptosis through inhibition of VEGF, isolated granulosa cells were cultured with control medium and 100ng/ml TSP-1 plus 0, 1, 10, 100 or 1000ng/ml VEGF for 48h. To investigate the effect of TSP-1 on various factors by western blotting isolated granulosa cells were cultured in 1.5ml medium containing  $3 \times 10^5$  cells/ml. Following overnight preincubation in a sterile humidified air atmosphere with 5% CO<sub>2</sub> at 37°C, prewarmed 1.5ml medium containing with 0, 1, 10, 100 or 1000ng/ml TSP-1 was added to each well for 96h, with medium refreshed every 48h. All treatments were carried out in triplicate and experiments were repeated three times.

#### **4.2.3.1 Caspase-3 and -7 activity assay**

To measure caspase-3 and -7 activities in isolated granulosa cells, the Caspase-Glo 3/7 assay was performed according to the manufacturer's instructions, 48h after treating the cells (see Chapter 2). Treatments were carried out in triplicate and experiments were repeated three times to reduce the possible effects of biological variability. As serum can generate a background caspase activity signal, an additional two wells contained cell culture medium and carrier solution without any cells and TNF $\alpha$  was used as a positive control.

#### **4.2.3.2 Western Blotting**

Protein was extracted from isolated granulosa cells as described previously (Chapter 2) and they were resolved on 4–20% Tris-glycine gels and transferred onto a PVDF-fl. Membranes were blocked for 1h at 25°C in Rockland Near Infra Red blocking buffer and incubated overnight at 4°C in blocking buffer containing rabbit anti-phospho-p42/44 ERK (1:1000), rabbit anti-phospho-p38 (1:500), FOXO1 (1:1000),

pTie-2 (1:1000) or HIF1 $\alpha$  (1:500) and  $\beta$ -actin (1:1000). After washing in PBS containing 0.5% Tween, membranes were incubated for 1h at 25°C in the dark in donkey anti goat IgG conjugated to IRDYE 800 and donkey anti rabbit IgG conjugated to Alexa Fluor 680 both at a dilution of 1:5000 in blocking buffer. Membranes were washed and proteins visualised and quantified using an Odyssey Infrared Imaging System. Sample loading was standardized to expression of  $\beta$ -actin.

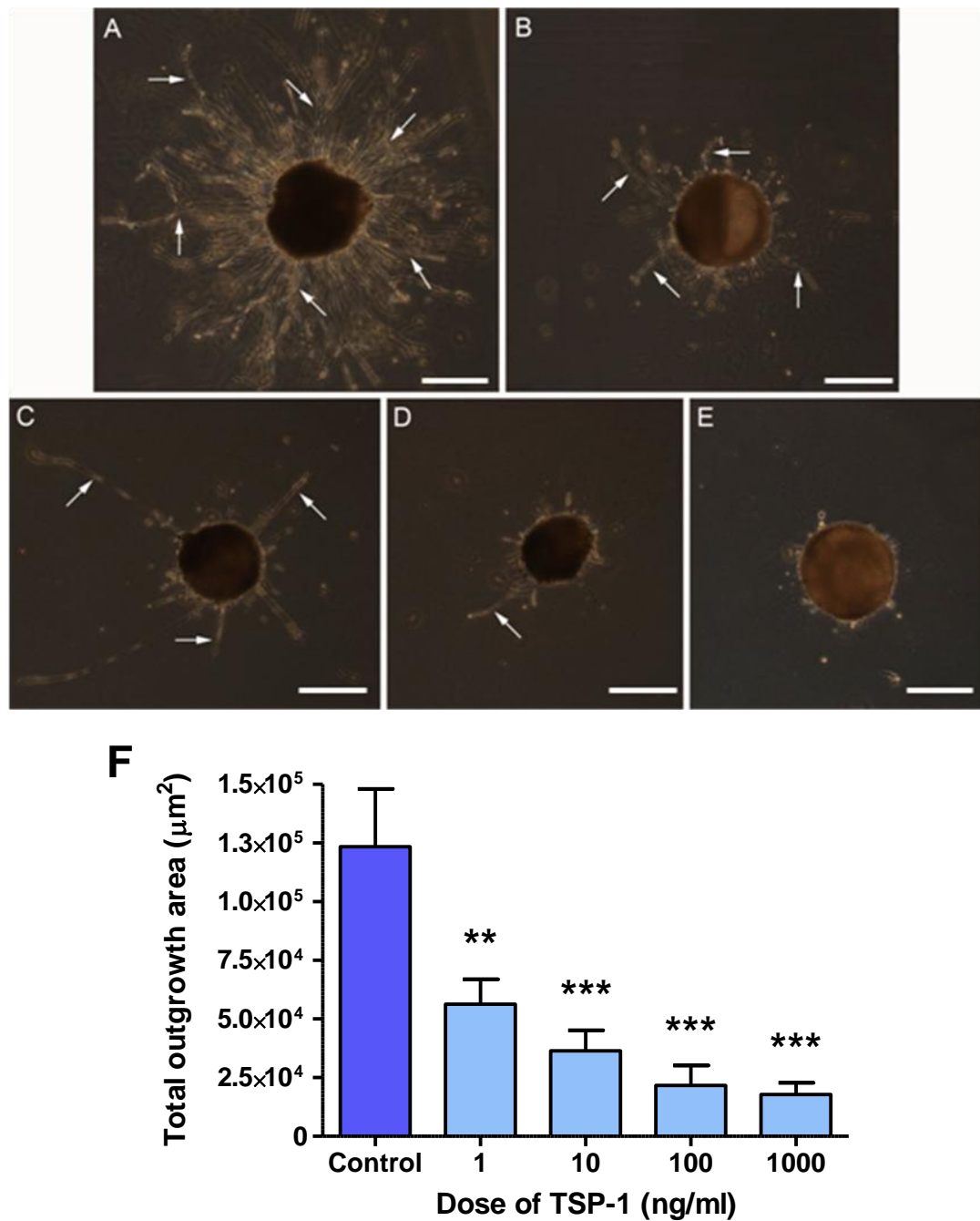
#### **4.2.4 Statistical Analyses**

Data are presented as mean  $\pm$  SEM and all results were analysed statistically using Graph Pad Prism 5 software (Graph Pad Software). The results in this chapter were analysed by means of a One-way ANOVA with a Bonferroni post-hoc test performed if the 95% confidence interval was reached to determine significance between all of the data groups. P values  $<0.05$  were accepted as statistically significant.

## 4.3 Results

### 4.3.1 Effect of TSP-1 on angiogenesis

To investigate the effect of TSP-1 on follicular angiogenesis, preantral/early antral follicles were cultured in serum-free medium treated with 1, 10, 100 and 1000ng/ml TSP-1 for 6 days. Treatment with TSP-1 resulted in a significant inhibition of follicular angiogenesis, in a dose dependent manner, (Figure 4.1) ( $P<0.01$ ), showing that TSP-1 is able to inhibit angiogenesis in whole ovarian follicles *in vitro*.

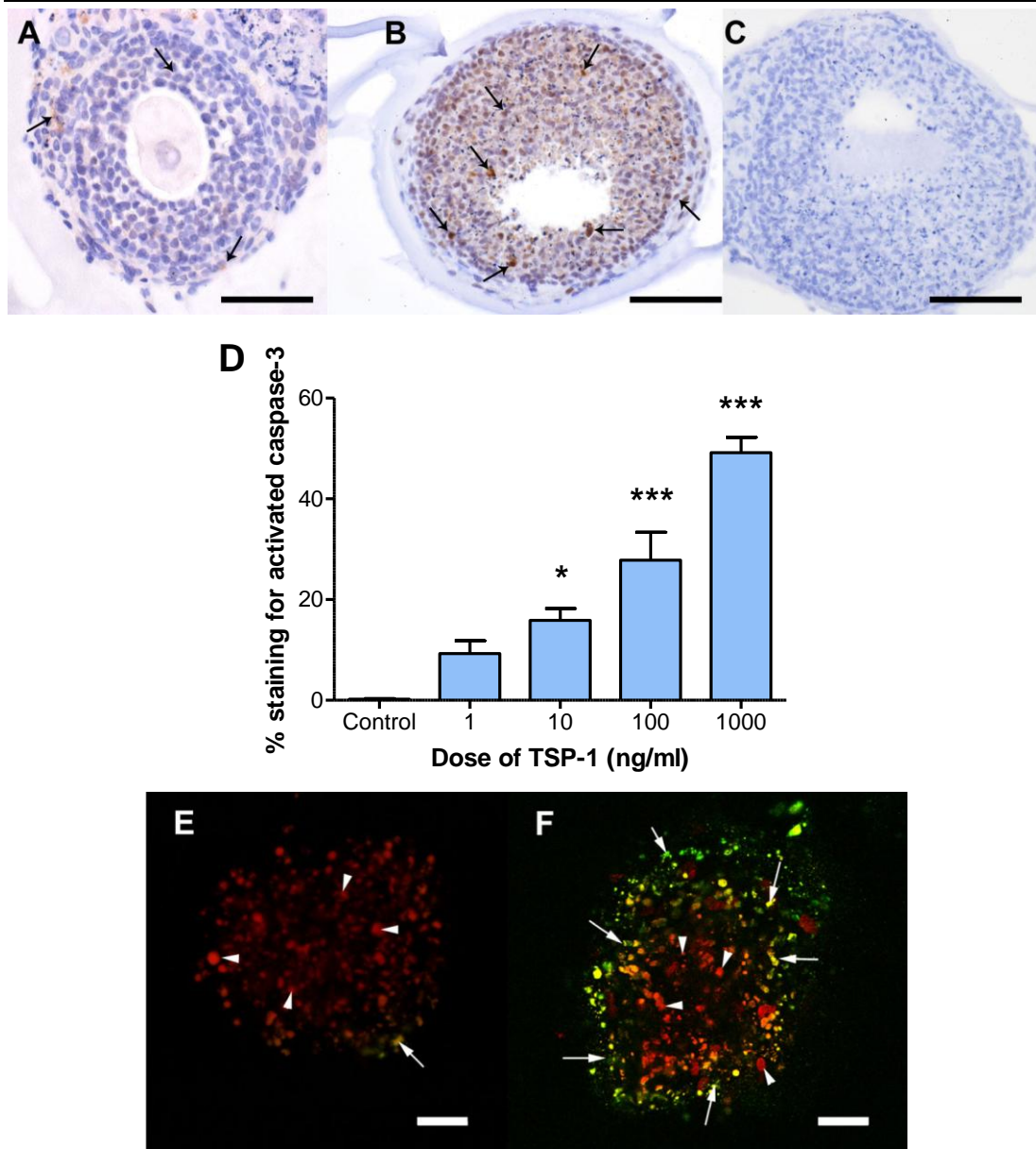


**Figure 4.1 Effect of TSP-1 on angiogenesis**

To determine the effect of TSP-1 on angiogenesis isolated preantral and early antral follicles were cultured with varying doses of TSP-1. At the end of the culture period the area of the angiogenic outgrowths was measured using Image Pro-Plus software. Representative images show endothelial outgrowths from a control follicle (n=25) (A) and follicles after treatment with TSP-1 at 1ng/ml (n=31) (B), 10ng/ml (n=28) (C), 100ng/ml (n=29) (D) or 1000ng/ml (n=36) (E). Results were analysed statistically using a One-way ANOVA with a Bonferroni post-hoc test to compare the TSP-1 treated follicles to the control follicles. Treatment with TSP-1 resulted in a significant dose-dependent decrease in outgrowth area (F). \*\* indicates  $P < 0.01$ , \*\*\* indicates  $P < 0.001$ , in comparison with the Control. White arrows indicate the endothelial outgrowths. Scale bars represent 200μm. Experiments were repeated in triplicate.

### **4.3.2 Effect of TSP-1 on follicular atresia**

To investigate the effect of TSP-1 on follicular apoptosis *in vitro*, preantral/early antral follicles that were cultured with 1, 10, 100 and 1000ng/ml TSP-1 for 6 days were then fixed, sectioned and stained for activated caspase-3. Representative images show a control follicle (Figure 4.2A) and a follicle treated with TSP-1 (Figure 4.2B), stained for activated caspase-3. A negative control for the immunohistochemistry is represented in Figure 4.2C. A dramatic increase in the level of staining for activated caspase-3 can be seen with TSP-1 treatment, and quantification of this staining indicates a significant increase in levels of follicular atresia in the presence of TSP-1 ( $P<0.05$ ) (Figure 4.2D). To confirm these findings, TUNEL labelling was performed. Representative images show a control follicle (Figure 4.2E) and a TSP-1 treated follicle (Figure 4.2F) labelled for TUNEL. A dramatic increase in cell death, shown by the green fluorescence, was observed after treatment with TSP-1.

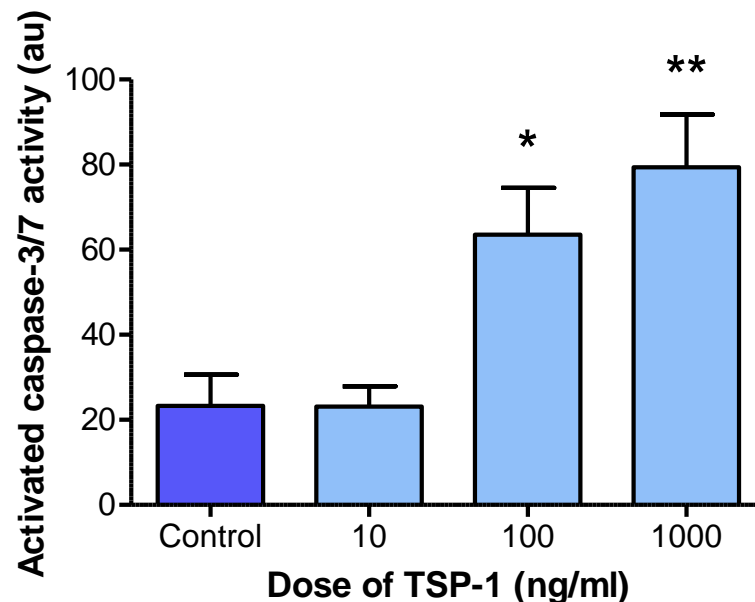


**Figure 4.2 Effect of TSP-1 on follicular atresia**

To determine the effect of TSP-1 on angiogenesis isolated preantral and early antral follicles were cultured with varying doses of TSP-1. At the end of the culture period the follicles were fixed and analysed for the expression of apoptotic markers. Fixed preantral/early antral follicles were sectioned and stained for activated caspase-3 (brown) and counterstained with haematoxylin (blue) and representative sections show a control follicle (A) and a follicle treated with TSP-1 (B), stained for activated caspase-3. A negative control for the immunohistochemistry is shown in C. These results were quantified (D) and analysed using a One-way ANOVA with a Bonferroni post-hoc test to compare TSP-1 treated follicles to the control follicles. Treatment with TSP-1 resulted in a significant dose-dependent increase in staining for activated caspase-3. \* indicates  $P < 0.05$ , \*\*\* indicates  $P < 0.001$ , in comparison with the Control. Follicles were also stained for TUNEL to confirm the presence of apoptotic cells and a representative control follicle (E) and a TSP-1 treated follicle (F) are shown. Black arrows indicate the expression of activated caspase-3, white arrows indicate apoptotic cells and white arrowheads indicate healthy cells. Scale bars represent  $50\mu\text{m}$ . Experiments were repeated in triplicate.

### 4.3.3 Effect of TSP-1 on granulosa cell apoptosis

It was proposed that TSP-1 could be acting directly on granulosa cells to induce apoptosis and this was investigated by culturing isolated granulosa cells with 0, 10, 100 and 1000ng/ml of TSP-1 for 48h followed by quantification of apoptosis using a luminescent caspase-3/7 assay. There was a significant increase in activated caspase-3/7 activity in granulosa cells treated with increasing doses of TSP-1 ( $P<0.05$ ) (Figure 4.3), showing that TSP-1 does act directly on granulosa cells to induce apoptosis.



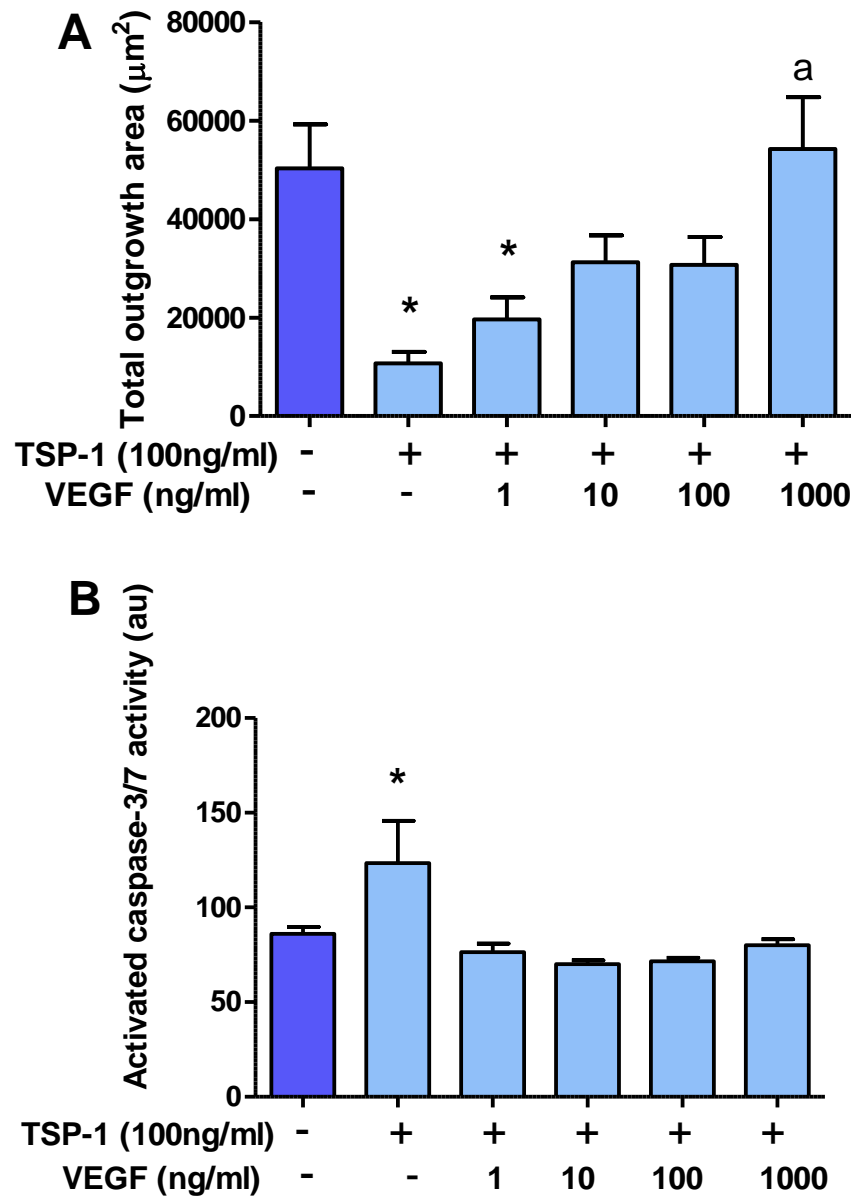
**Figure 4.3 Effect of TSP-1 on granulosa cell apoptosis**

To determine the effect of TSP-1 on apoptosis granulosa cells were isolated from preantral and early antral follicles and they were cultured with varying doses of TSP-1 ( $n=4$  per treatment). At the end of the culture period these granulosa cells were analysed for expression of activated caspase-3/7 activity using a luminescent caspase activity assay, as an indicator of follicle health. Results were analysed statistically using a One-way ANOVA with a Bonferroni post-hoc test to compare the TSP-1 treated granulosa cells to the control granulosa cells. Treatment with TSP-1 resulted in a significant dose-dependent increase in activated caspase-3 expression \* indicates  $P<0.05$ , \*\* indicates  $P<0.01$ , in comparison to the control. Luminescence is given in arbitrary units (au). Experiments were repeated in triplicate.

#### **4.3.4 Effect of TSP-1 and VEGF co-culture on angiogenesis**

To investigate whether the addition of VEGF to the culture could attenuate the anti-angiogenic effect of TSP-1, preantral/early antral follicles were cultured in serum-free medium in the presence of 100ng/ml TSP-1 and either 0, 1, 10, 100 or 1000ng/ml VEGF for 6 days. Figure 4.4A shows that follicles cultured with both TSP-1 alone and TSP-1 plus 1ng/ml VEGF had significantly reduced follicular angiogenesis compared with controls ( $P<0.05$ ). However, increasing concentrations of VEGF restored follicular angiogenesis to control levels ( $P<0.05$ ).





**Figure 4.4** Effect of TSP-1 and VEGF co-culture on angiogenesis (A) and on granulosa cell apoptosis (B; activation of caspase-3/7)

Preantral/early antral follicles were cultured with either 100ng/ml TSP-1 ( $n=29$ ) alone, with 100ng/ml TSP-1 plus 1 ( $n=33$ ), 10 ( $n=34$ ), 100 ( $n=35$ ) or 1000ng/ml VEGF ( $n=33$ ) (A). Addition of VEGF to follicles cultured with TSP-1 led to a reduction in the anti-angiogenic effect of TSP-1, in a dose dependent manner. VEGF at all dose levels prevented the TSP-1 induced increase in caspase-3/7 activity (B). Results were analysed statistically using a One-way ANOVA with a Bonferroni post-hoc test to compare all the different treatment groups to their respective control (no TSP-1 or VEGF). \* indicates  $P<0.05$ , in comparison with respective control value; <sup>a</sup> indicates  $P<0.05$ , in comparison with TSP-1 alone or with TSP-1 + 1ng/ml VEGF. Luminescence is given in arbitrary units (au).

### **4.3.5 Effect of TSP-1 and VEGF co-culture on granulosa cell apoptosis**

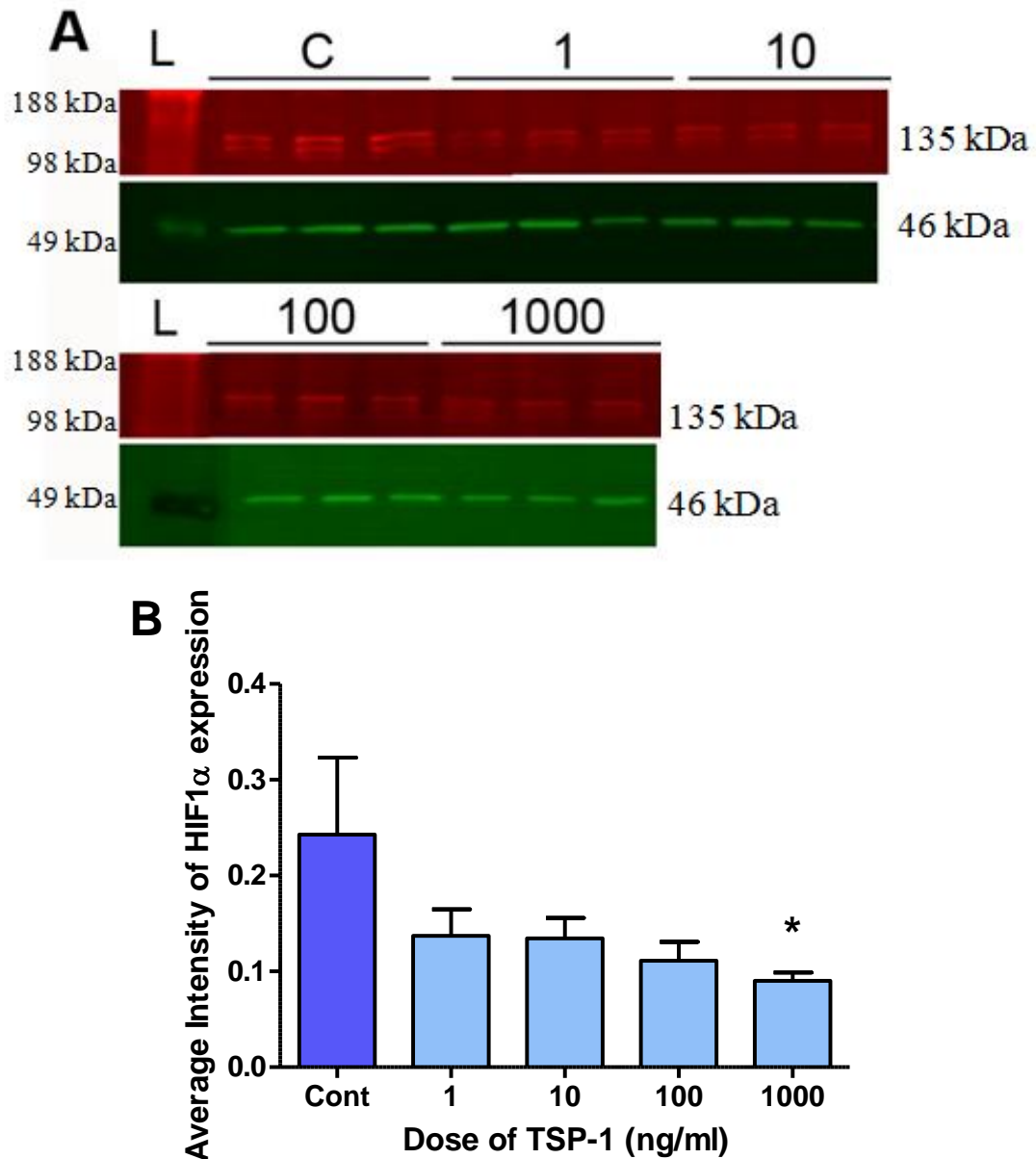
Having shown that treatment with VEGF can rescue the inhibition of angiogenesis by TSP-1, it was hypothesised that the inhibition of VEGF activity by TSP-1 could also contribute to its ability to induce follicular atresia. To investigate this, isolated granulosa cells were cultured with both TSP-1 and VEGF. As 100ng/ml TSP-1 was shown to induce apoptosis in isolated granulosa cells (Figure 4.3), the granulosa cells were cultured with 100ng/ml TSP-1, alone or in combination with either 1, 10 or 100ng/ml VEGF for 48h, followed by quantification of caspase-3/7 activity. Figure 4.4B shows that granulosa cells cultured with 100ng/ml TSP-1 alone had significantly increased levels of activated caspase-3/7 activity ( $P<0.05$ ) in comparison to granulosa cells cultured in control medium. Addition of all concentrations of VEGF in combination with TSP-1 resulted in the reduction of caspase-3/7 activity to control levels, suggesting that VEGF is able to rescue granulosa cells from the apoptosis that is induced by TSP-1.

### **4.3.6 Mechanisms of anti-angiogenesis**

#### ***4.3.6.1 Quantification of HIF1 $\alpha$ expression by western blotting***

Angiogenesis is stimulated under hypoxic conditions and one of the main compounds involved in stimulating hypoxia-induced angiogenesis is hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ). As conditions become more hypoxic levels of HIF1 $\alpha$  rise and this stimulates the production of VEGF, resulting in the induction of angiogenesis (Berra et al., 2000, Mukhopadhyay and Datta, 2004, Liao and Johnson, 2007, Fong, 2009). As TSP-1 has been shown to inhibit angiogenesis and also to act, at least in part, via

interaction with VEGF it was investigated whether treatment with TSP-1 would affect the expression of HIF1 $\alpha$ . To investigate this isolated granulosa cells were treated with 0, 1, 10, 100 and 1000ng/ml of TSP-1 for 48h followed by quantification of the expression of HIF1 $\alpha$  by western blotting (Figure 4.5) and there was a decrease in the expression of HIF1 $\alpha$  after treatment with TSP-1, which was significant ( $P<0.05$ ) for the 1000ng/ml treatment group.



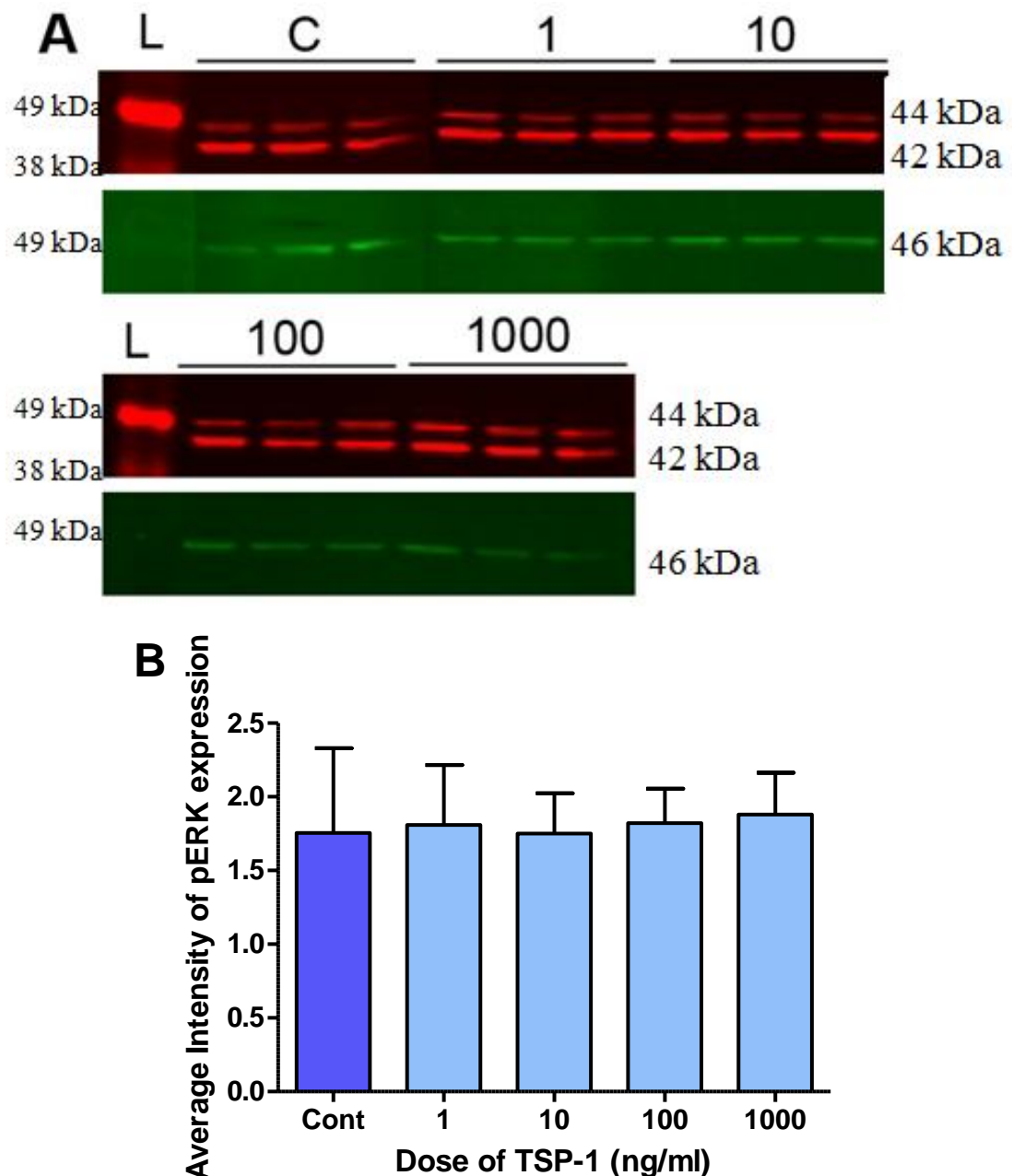
**Figure 4.5 Levels of HIF1 $\alpha$  expression after treatment with TSP-1**

Isolated granulosa cells were cultured with varying doses of TSP-1 ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed. To attempt to determine a potential mechanism of anti-angiogenesis the level of hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with TSP-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically using a One-way ANOVA with a Bonferroni post-hoc test to compare the TSP-1 treated granulosa cells to the control granulosa cells. The results show that there is a decrease in the expression of HIF1 $\alpha$  after treatment with TSP-1 that is statistically significant for granulosa cells cultured in 1000ng/ml TSP-1 when compared to the control (\* $P<0.05$ ). Experiments were repeated in triplicate.

### **4.3.7 Mechanisms of induction of apoptosis**

#### ***4.3.7.1 Quantification of pERK expression by western blotting***

As this chapter has shown that TSP-1 is able to induce apoptosis further investigations were performed to establish the mechanisms by which TSP-1 can induce apoptosis. Phospho-ERK (pERK) plays an important role in anti-apoptotic/pro-survival pathways and it is possible that TSP-1 could be inhibiting pERK expression, resulting in the induction of apoptosis. To investigate this isolated granulosa cells were cultured with 0, 1, 10, 100 and 1000ng/ml of TSP-1 for 48h followed by quantification of the expression of pERK by western blotting (Figure 4.6). The results showed that there was no difference in expression of pERK after treatment with TSP-1.

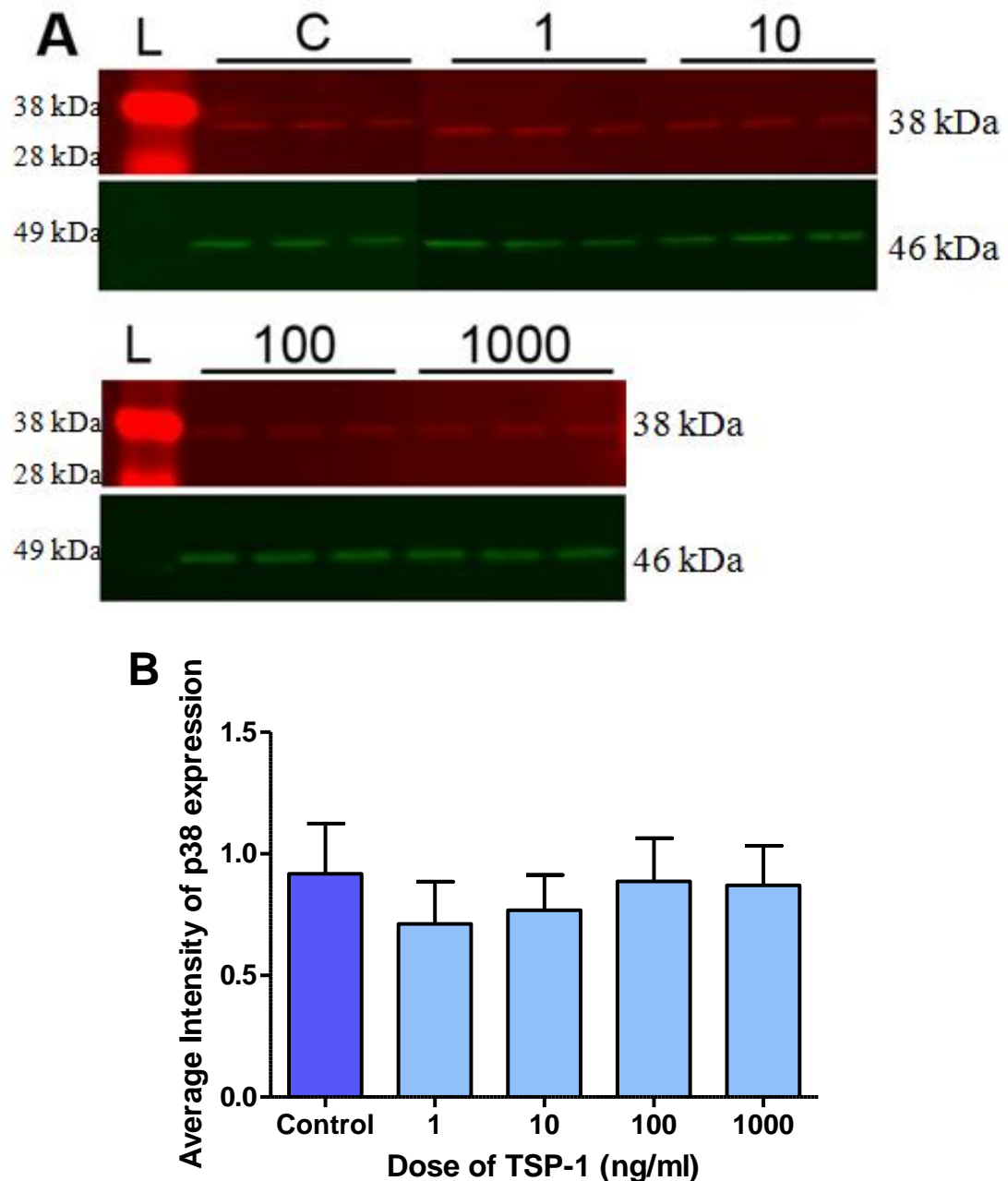


**Figure 4.6 Levels of pERK expression after treatment with TSP-1**

Isolated granulosa cells were cultured with varying doses of TSP-1 ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed. To try to determine a potential protein mechanism of apoptosis induction the level of pERK expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with TSP-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically using a One-way ANOVA to compare the TSP-1 treated granulosa cells to the control granulosa cells. The results show that there is no change in the expression of pERK after treatment with TSP-1. Experiments were repeated in triplicate.

#### **4.3.7.2 Quantification of p38 expression by western blotting**

As there was no difference in the expression of the pro-survival factor pERK after treatment with TSP-1 the expression of the pro-apoptotic factor p38 was investigated. Isolated granulosa cells were treated with 0, 1, 10, 100 and 1000ng/ml of TSP-1 for 48h followed by quantification of the expression of p38 by western blotting (Figure 4.7). The results showed that there was no difference in expression of p38 after treatment with TSP-1.



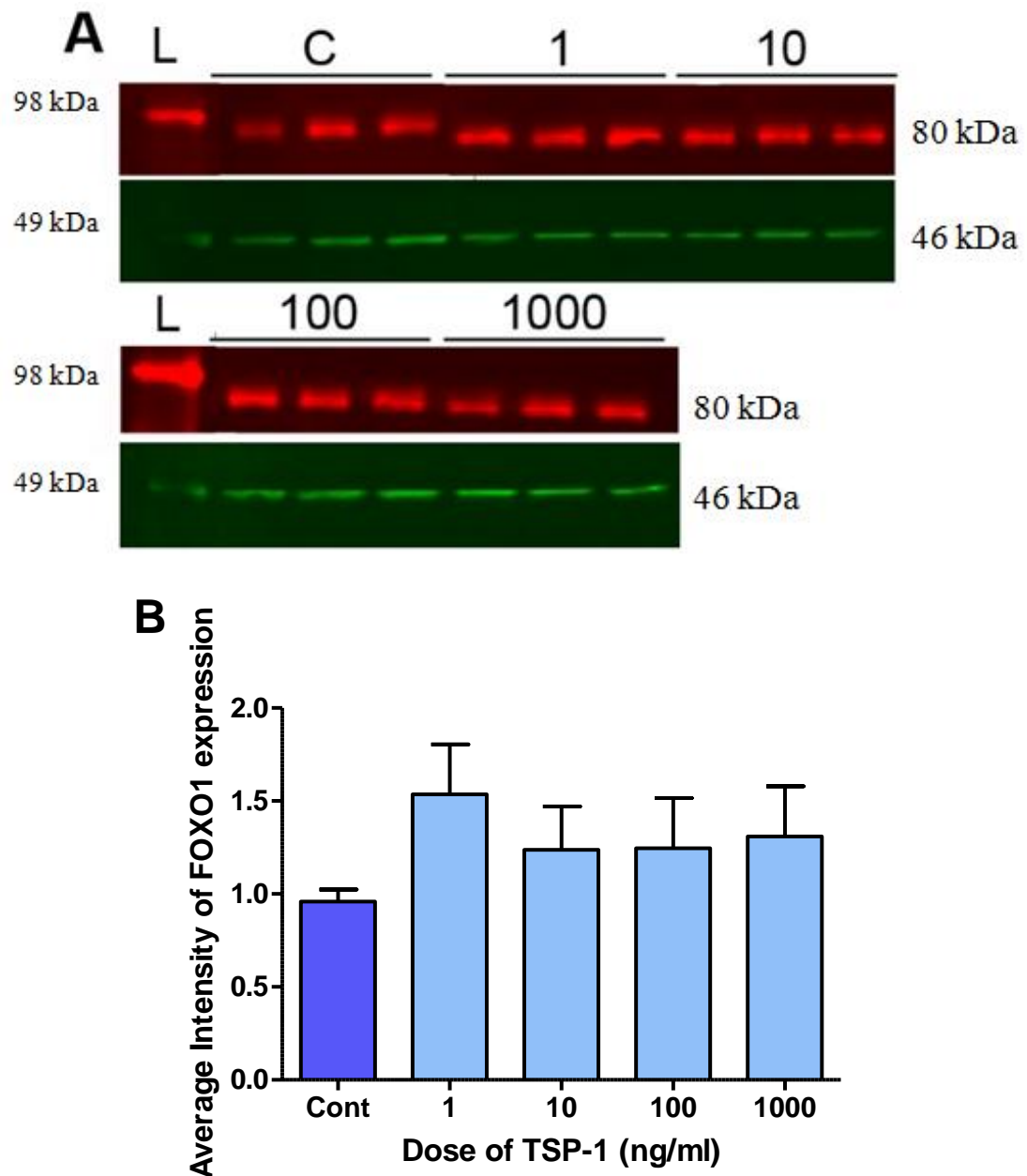
**Figure 4.7 Levels of p38 expression after treatment with TSP-1**

Isolated granulosa cells were cultured with varying doses of TSP-1 ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed. To try to determine a potential mechanism of apoptosis induction the level of p38 expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with TSP-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically using a One-way ANOVA to compare the TSP-1 treated granulosa cells to the control granulosa cells. The results show that there is no change in the expression of p38 after treatment with TSP-1. Experiments were repeated in triplicate.



#### **4.3.7.3 Quantification of FOXO1 expression by western blotting**

FOXO1 is a transcription factor that blocks cell division and promotes apoptosis and as FOXO1 has been shown to be expressed on granulosa cells it was investigated whether this could be involved in the induction of apoptosis after treatment with TSP-1. To investigate this isolated granulosa cells were treated with 0, 1, 10, 100 and 1000ng/ml of TSP-1 for 48h followed by quantification of the expression of FOXO1 by western blotting (Figure 4.8). The results showed that there was an increase in the expression of FOXO1 in granulosa cells treated with TSP-1; however this did not reach statistical significance.



**Figure 4.8 Levels of FOXO1 expression after treatment with TSP-1**

Isolated granulosa cells were cultured with varying doses of TSP-1 ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed. To try to determine a potential mechanism of apoptosis induction the level of FOXO1 expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with TSP-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically using a One-way ANOVA to compare the TSP-1 treated granulosa cells to the control granulosa cells. A trend in increasing expression of FOXO1 can be seen in granulosa cells treated with TSP-1 although results were not statistically significant. Experiments were repeated in triplicate.

## 4.4 Discussion

In this chapter the *in vitro* angiogenesis assay described in Chapter 3 was utilised to demonstrate that TSP-1 is a potent inhibitor of follicular angiogenesis. Furthermore, it has been shown that TSP-1 promotes follicular atresia *in vitro* by directly inducing apoptosis of granulosa cells.

TSP-1 is an endogenous angiogenesis inhibitor and as it is most highly expressed during the preantral and early antral stages of follicular development, when vascularisation is occurring, it is likely that TSP-1 acts to limit any overgrowth of the vasculature that develops in response to the high levels of pro-angiogenic factors expressed at these stages. VEGF is one of the main factors involved in the stimulation of angiogenesis and TSP-1 has been shown to bind to VEGF and inhibit its action (Greenaway et al., 2007), suggesting that the anti-angiogenic action of TSP-1 could, at least in part, be regulated through interaction with VEGF.

This possibility was investigated by culturing granulosa cells with a combination of TSP-1 and VEGF. The results showed that treatment with VEGF attenuated the TSP-1-induced inhibition of angiogenesis, suggesting that TSP-1 inhibits angiogenesis via the inhibition of VEGF activity. In the presence of TSP-1, CD36 can interact with VEGFR2 and form receptor-complexes that can inhibit the phosphorylation of VEGFR2 by VEGF (Zhang et al., 2009). This suggests that the role of TSP-1 in inhibiting angiogenesis is modulated both through its receptors and through interaction with VEGF, but also that the formation of receptor complexes could play

a role in regulating the function of TSP-1. The mechanisms of TSP-1-induced inhibition of angiogenesis were investigated by quantifying the levels of some of the factors involved in the angiogenic cascade. As the production of HIF1 $\alpha$  is one of the main triggers for the induction of angiogenesis (Ferrara, 2005) it was suggested that TSP-1 could be acting on HIF1 $\alpha$ . This study has shown that treatment with TSP-1 reduces the expression of HIF1 $\alpha$ , in a dose dependent manner. This would lead to reduced production of pro-angiogenic factors such as VEGF and the inhibition of angiogenesis, suggesting that one of the mechanisms by which TSP-1 inhibits angiogenesis is via the suppression of HIF1 $\alpha$ . This, along with the inhibition of the action VEGF, would constrain the initiation of angiogenesis and impede the development of new blood vessels.

It has previously been shown, in a descriptive study, that TSP-1 expression is up-regulated during follicular atresia in the marmoset ovary (Thomas et al., 2008). It was proposed that this phenomenon would also occur in the rat ovary and this chapter has confirmed that TSP-1 is expressed in the granulosa cells of late preantral and antral follicles in the rat ovary. Since CD36 is expressed in granulosa cells, it was hypothesised that TSP-1 may be acting directly on these cells in an autocrine fashion to promote follicular atresia via an apoptotic mechanism (Thomas et al., 2008). Moreover, an extravascular role for TSP-1 has been suggested (Jimenez et al., 2000, Nor et al., 2000, Greenaway et al., 2007) and, using a granulosa cell line, Greenaway and colleagues showed that TSP-1 treatment decreased VEGF levels and rendered cells more susceptible to TNF- $\alpha$ -induced apoptosis (Greenaway et al., 2007). Therefore it was investigated whether treatment with TSP-1 would be able to

induce follicular atresia. This chapter has shown for the first time that TSP-1 directly induces apoptosis of primary granulosa cells via the activation of caspase-3. This effect on granulosa cell apoptosis occurred both in intact follicles and isolated granulosa cells. TSP-1 acts via the receptor CD36 and since CD36 receptors are present on granulosa cells, it is likely that the promotion of follicular apoptosis by TSP-1 is due to a direct effect on the granulosa cells.

In addition, VEGF has been shown to have a cytoprotective role in the ovary (Greenaway et al., 2004) and to promote cell survival. As TSP-1 binds to VEGF and inhibits its action (Greenaway et al., 2007), it was proposed that TSP-1 could be inducing apoptosis via the inhibition of the survival-promoting effects of VEGF. The present study has shown that the addition of VEGF to the granulosa cell cultures was sufficient to rescue the cells from the pro-apoptotic effects of TSP-1, suggesting that TSP-1 may act via the inhibition of VEGF action. However, since TSP-1 is capable of directly activating pro-apoptotic pathways in other cell types (Guo et al., 1997, Jimenez et al., 2000, Nor et al., 2000), a direct mechanism of action of TSP-1 in granulosa cells cannot be ruled out. As VEGF is a principal factor involved in follicular angiogenesis and development (Wulff et al., 2001a, Wulff et al., 2001b, Wulff et al., 2002, Ferrara, 2004, Fraser et al., 2006), it may be that addition of VEGF to the granulosa cells is sufficient to override the negative effects of TSP-1, regardless of the mechanism of action of TSP-1. As TSP-1 induces apoptotic signalling in other cell types via CD36 (Dawson et al., 1997, Guo et al., 1997), and we have shown that VEGF can inhibit the pro-apoptotic effect of TSP-1, it is

possible that TSP-1 induces apoptosis in the ovary by two mechanisms; directly via CD36 signalling and indirectly via the inhibition of VEGF.

The mechanisms by which TSP-1 can induce apoptosis were further investigated by quantifying the expression of pro- or anti-apoptotic factors in isolated granulosa cells. It was anticipated that TSP-1 could have an effect on the expression of pERK and/or p38 (Donnini et al., 2004, Nakagawa et al., 2005, Ridnour et al., 2005) as they are key factors involved in anti- and pro-apoptotic pathways, respectively, that either lead to the inhibition or induction of the caspase cascade. As treatment with TSP-1 increases follicular atresia via activated caspase-3 signalling, one of the main molecules involved in the induction of apoptosis, it is likely that TSP-1 also regulates pro- or anti-apoptotic pathways further upstream. There was no difference in the expression of the pro-survival factor pERK after treatment with TSP-1. This suggests that the mechanism by which TSP-1 induces follicular atresia is not due to the inhibition of pro-survival pathways but could be due to stimulation of pro-apoptotic pathways. It was observed that there was a trend for increasing expression of p38 after treatment with TSP-1, suggesting that TSP-1 may induce follicular atresia through the induction of pro-apoptotic pathways.

In addition, FOXO1 is a transcription factor that is involved in the induction of apoptosis and it has been shown to increase the level of CD36 expression in cell membranes (Bastie et al., 2005, Nahle et al., 2008). This suggests that FOXO1 may play a role in the induction of apoptosis induced by treatment with TSP-1. The expression of FOXO1 after treatment with TSP-1 was quantified to further elucidate

the mechanisms of TSP-1-induced follicular atresia but there was no difference in the expression of FOXO1. As there was no significant change in the expression of key factors involved in both pro- and anti-apoptotic pathways after treatment with TSP-1, other than activated caspase-3, this suggests that the regulation of TSP-1-induced follicular atresia is complex and involves a delicate balance between pro- and anti-apoptotic pathways. However, expression of activated caspase-3 was significantly increased, and there was a trend of increasing expression of p38, after treatment with TSP-1 which suggests that TSP-1-induced follicular atresia could be due to the induction of pro-apoptotic pathways rather than the inhibition of pro-survival pathways.

In summary, this chapter has shown for the first time that TSP-1 inhibits angiogenesis in the ovarian follicle and has a functional role in the induction of follicular atresia. In addition, it has been demonstrated that TSP-1 directly promotes apoptosis in primary granulosa cells, indicating an extravascular role for TSP-1 during follicular development. Increased angiogenesis and decreased follicular atresia are hallmarks of PCOS (Agrawal et al., 1998a, Franks et al., 2000), therefore the use of TSP-1 as a therapeutic for this disorder is an exciting possibility. TSP-1 treatment could potentially have a dual effect by targeting the abnormal ovarian angiogenesis in PCOS, as well as facilitating the atresia of abnormal follicles via induction of apoptosis. To support this hypothesis the effects of a small peptide mimetic of TSP-1 were analysed using an *in vivo* marmoset model to confirm that these effects would occur *in vivo* as well as *in vitro*.

**Chapter 5 A Thrombospondin-1-  
mimetic Peptide, ABT-898, Suppresses  
Angiogenesis and Promotes Follicular  
*Atresia in vivo***



## **5 Chapter 5 A Thrombospondin-1-mimetic Peptide, ABT-898, Suppresses Angiogenesis and Promotes Follicular Atresia *in vivo***

### **5.1 Introduction**

Angiogenesis is a complex process that is tightly regulated by pro- and anti-angiogenic factors such as VEGF and TSP-1 (Tolsma et al., 1993, Stouffer et al., 2001, Armstrong and Bornstein, 2003, Folkman, 2004, Fraser and Duncan, 2005, Folkman, 2007, Fraser and Duncan, 2009). The action of pro-angiogenic factors is offset by the action of anti-angiogenic factors, although the importance of a balance between these factors in regulating physiological angiogenesis is largely unknown.

Angiogenesis is a key feature of many pathological conditions and polycystic ovary syndrome (PCOS) is characterised by aberrant angiogenesis (Agrawal et al., 2002), hyperandrogenism, and the accumulation of small anovulatory antral follicles with reduced follicular atresia (Franks et al., 2000). PCOS is the most common endocrine disorder in women of reproductive age and existing treatments for PCOS are limited. They are either aimed at controlling symptoms by changing hormone levels within the body or cauterisation of PCOS ovaries can be carried out, resulting in a period where ovarian cycles become ovulatory. This effect is partly due to the destruction of accumulated non-ovulatory follicles. There is currently no therapy that specifically targets abnormal ovarian angiogenesis in PCOS, or that facilitates the destruction of accumulated antral follicles. Therefore, a novel therapy with a dual effect of

inhibiting angiogenesis and inducing atresia could be of benefit in treating PCOS, especially if it did not interfere with ovulation of dominant follicles.

It has been demonstrated that there is a decrease in both TSP-1 and CD36 expression during follicular development (Petrik et al., 2002, Greenaway et al., 2005), therefore TSP-1 signalling may be involved in restricting angiogenesis in early follicular development (Fraser et al., 2006). In addition, it has been observed that TSP-1 mRNA and protein is up-regulated during follicular atresia *in vivo* (Thomas et al., 2008), suggesting that TSP-1 may be involved in the cessation of angiogenesis in follicles undergoing atresia. In a subsequent study, a functional role for TSP-1 in the inhibition of follicular angiogenesis and promotion of follicular atresia has been demonstrated, using a novel *in vitro* angiogenesis assay (Chapter 4). In the ovaries of the TSP-1 null mouse, there is an increase in follicle number which may reflect a reduced rate of follicular atresia (Lawler et al., 1998). TSP-1 has been reported to induce apoptosis in cultured cells derived from the vascular endothelium (Guo et al., 1997), suggesting that this factor may inhibit angiogenesis by promoting endothelial cell degradation (Jimenez et al., 2000). Further to this, in the previous chapter it has been shown that TSP-1 promotes apoptosis of isolated granulosa cells, thus it is likely that the promotion of follicular atresia by TSP-1 involves a direct effect on the granulosa cells (Garside et al., 2010).

TSP-1 is a large multifunctional glycoprotein, and its anti-angiogenic activity has been isolated to a smaller sequence within TSP-1 (TSR-1) (Zhang and Lawler, 2007). A much smaller heptapeptide sequence within TSR-1, GVITRIR, has been

mimicked by the modified nonapeptide, ABT-510: Ac-Sar-GV-DalloIle-T-Nva-IRP-ethylamide, which has been shown to be active anti-angiogenically in slowing tumour growth in preclinical models (Anderson et al., 2007, Yang et al., 2007) and reduces VEGF production in a model of ovarian cancer (Greenaway et al., 2009). It has also been demonstrated to be efficacious in the treatment of spontaneously occurring cancer in companion dogs (Rusk et al., 2006), and has been in clinical trials, mainly as monotherapy (Hoekstra et al., 2005, Hoekstra et al., 2006, Gietema et al., 2006, Markovic et al., 2007). The thrombospondin mimetic peptide-A-428898 (ABT-898) (Abbott Laboratories, N. Chicago, IL, USA), a substituted octapeptide Ac-GV-DalloIle-SQIRP-ethylamide, is a second generation mimetic of the anti-angiogenic activity of TSP-1, having greatly increased potency over that of ABT-510 as well as slower clearance in primates. ABT-898 is thus expected to have greater efficacy than the other available TSP-1-mimetic peptides.

In this chapter the effect of ABT-898 on follicular angiogenesis was investigated using the *in vitro* angiogenesis assay described in Chapter 3, prior to studying its effects *in vivo* in the common marmoset (*Callithrix jacchus*). The present study first determined the inhibitory effect of ABT-898 on follicular angiogenesis and survival *in vitro*. Subsequently, *in vivo* treatment during the follicular phase in marmosets was studied to confirm a functional role for this peptide in a physiological setting.

## 5.2 Materials and Methods

### 5.2.1 Follicle Culture

For all experiments, preantral/early antral follicles were cultured individually in flat-bottomed 12-well plates, embedded in the centre of a 20µl droplet of growth-factor reduced Matrigel. The Matrigel provides a three-dimensional extracellular matrix support for follicle growth and allows the vascular outgrowths to develop in three-dimensions, as they would *in vivo*. Follicles were incubated for 6 days in a sterile humidified air atmosphere with 5% CO<sub>2</sub> at 37°C in 600µl serum-free EBM-2 medium supplemented with BSA (0.1%), L-glutamine (3mM), penicillin (100 IU/ml), streptomycin (0.1mg/ml), transferrin (2.5µg/ml), selenium (4ng/ml), insulin (10ng/ml) and L-ascorbic acid, sodium salt (50µg/ml), with culture medium refreshed every 48h.

In order to test the effects of ABT-898, follicles were randomly placed into groups of 6 and cultured in control medium or in the presence of 0.1, 1, 10, 100 or 1000ng/ml ABT-898 (A-428898 amino acid sequence N-Acetyl-glycine-valine-D-alloisoleucine-serine-glutamine-isoleucine-arginine-proline-ethylamide, Abbott Laboratories) and the experiment was carried out twice.

#### 5.2.1.1 Analysis of Angiogenic Sprouting

To assess the impact of the treatments on angiogenic sprouting, follicles were examined and photographed directly in the 12-well plates under an inverted light microscope, at x10 magnification, with Axiovision image capturing software on days

0 and 6 of the culture period. The total area of angiogenic sprouting for each follicle was determined using Image Pro Plus software. This was done by drawing an area of interest around the outgrowths (but excluding the follicle), and the sum of the area of all sprouts was calculated by the software. The mean area of angiogenic sprouting for each treatment was then compared to the control.

### 5.2.2 Marmosets

Experiments were carried out in accordance with the Animals (Scientific Procedures) Act, 1986, and approved by the Local Ethical Review Process Committee. To synchronize follicular recruitment, selection and ovulation during treatment cycles, adult (2-4 years old) female common marmoset monkeys with a body weight of 350-400g and regular ovulatory cycles, housed as described previously (Chapter 2), were injected with 1µg prostaglandin PGF<sub>2α</sub> analogue (cloprostenol) per animal, i.m. on day 13-15 of the luteal phase to induce luteolysis. The day of prostaglandin injection was designated follicular d 0. This method of synchronizing follicular recruitment is followed by follicle selection on cycle d 5 and ovulation between d 9-11 (Summers et al., 1985, Gilchrist et al., 2001).

To determine the pharmacokinetics of ABT-898 in the marmoset, 4 adult females were given a single sc injection of 2.5mg/kg of ABT-898 dissolved in 5% glucose solution followed by sterile filtration using a Ministrat, (Sartorius AG, Gottingen, Germany). Blood samples (300µl) were collected from the femoral vein at 0, 0.25, 1, 2, 4, 6, 8, 12 and 24h. Plasma was stored at -20C until required for assays.

**5.2.2.1 Treatment with ABT-898 throughout the follicular phase**

To investigate the effect of ABT-898 on follicular angiogenesis and atresia *in vivo*, ABT-898 was administered to marmosets (n=5) by twice daily sc injections of 2.5mg/kg, prepared as above, on d0-9 of the follicular phase. Control marmosets (n=5) were treated with vehicle. On d10, of the follicular phase which corresponds to the peri-ovulatory period in controls, all animals were injected i.v. with 20mg BrdU in saline one hour before being sedated using 200µl ketamine hydrochloride and 200µl Saffan. After perfusion with 4% NBF, tissues were removed immediately, weighed, and fixed in 4% NBF for 24h. Plasma progesterone was measured throughout the study using assays described previously (Smith et al., 1990).

**5.2.2.2 Long-term *in vivo* treatment with ABT-898 depot**

To investigate the effect of long-term high-dose treatment with ABT-898 on ovulatory cycles, a depot formulation of 25mg ABT-898 in biodegradable microspheres was suspended in 500µl diluent and injected sc during the late luteal phase in 3 marmosets with regular ovulatory cycles. This was followed by a second injection 14 days later. The first injection was accompanied by treatment with a prostaglandin analogue to induce luteolysis. Thus, this treatment period was designed to cover the entire follicular phase and early luteal phase. The second injection was designed to cover the luteal phase in the event of ovulation occurring. Blood samples were collected three times per week for 3 cycles prior to treatment and for 3 months after the first injection.

### 5.2.3 Immunohistochemistry

The effects of the treatment on granulosa cell proliferation was determined by quantifying the number of proliferating cells stained for BrdU and the effect on vascular density was determined by localising endothelial cells using CD31. Dual staining for BrdU and CD31 was also performed to enable quantification of proliferating endothelial cells. Immunohistochemistry was performed as described previously (Chapter 2) using the primary antibodies CD31 (monoclonal, diluted 1:20 in TBS) or BrdU (monoclonal, diluted 1:30 in TBS). Incubation with the primary antibodies was carried out overnight at 4°C and incubation with the secondary antibody (rabbit antimouse Ig, 1:60 diluted in TBS) was for 40min at room temperature, followed by incubation of the APAAP complex (1:100 dilution in NRS and TBS) for 40min at room temperature. Visualisation was performed using NBT solution.

For detection of proliferating endothelial cells, dual staining was obtained by immunohistochemistry with CD31 and BrdU. For CD31 detection, the protocol was followed as described above but visualisation was performed with fast red. The second primary to BrdU was then added, diluted 1:5000 in NRS, and incubated overnight at 4°C. After post-incubation washes with TBS, a biotinylated rabbit anti-sheep secondary antibody was added, followed by ABC-AP. After incubation with the ABC-AP complex, slides were transferred to NBT buffer before staining with NBT for 15min.

**5.2.3.1 Caspase-3**

To assess follicular atresia, immunohistochemistry for activated caspase-3 was performed as described previously (Fraser et al., 2006, Thomas et al., 2007). To determine the localisation and changes in the number of dying cells, a rabbit antibody to activated caspase-3 was used. The sections were incubated overnight at 4°C with activated caspase-3 antibody at 1:100 dilution in NGS. Slides were then washed in TBS and incubated with labelled polymer-HRP as secondary antibody (rabbit EnVision kit) for 30mins. Visualisation was achieved by DAB Substrate.

**5.2.3.2 Quantification of immunohistochemistry**

Slides stained with H&E were used to assess morphology of the ovaries. Stages of follicular development were defined as previously reported (Chapter 2(Wulff et al., 2002, Thomas et al., 2007). Only those follicles with a visible oocyte containing a nucleus were considered to ensure proper follicular classification in these follicles. In addition, preovulatory follicles >2mm and early corpora lutea were identified and counted.

The extent of follicular atresia was investigated by analysis of H&E-stained sections and sections stained for activated caspase-3. In the initial analysis, H&E stained follicles were classed as atretic if they had more than 10% pyknotic nuclei and follicles stained for activated caspase-3 were classified as atretic if they had greater than 10% caspase-3 staining. This classification is based on previously described criteria for the measurement of follicular cell death (Byskov, 1974). Follicles with



morphological signs such as granulosa cell shrinkage and detachment of cells from the oocyte or basement membrane were also classed as atretic.

Granulosa cell proliferation was measured by counting both the number of BrdU-positive nuclei and the total number of nuclei in the selected fields. A proliferation index (i.e. BrdU-positive nuclei expressed as a percentage of the total number of nuclei) was calculated for each follicle. Relative vascularisation was quantified by measuring the total area of CD31 staining and the total area of the thecal layer so that the area of endothelial cells could be expressed as a percentage of total thecal area, using Image Pro Plus software. Endothelial cell proliferation was measured by counting the number of dual-stained cells (BrdU- and CD31-positive cells) and the total number of proliferating cells (i.e. BrdU-positive cells) in the thecal layer. The proportion of proliferating endothelial cells (dual-stained cells) was expressed as a percentage of the total number of proliferating cells. All vessels in the theca of each follicle were counted for quantification of relative vascularisation and endothelial cell proliferation.

For all of these quantifications early preantral, preantral and antral follicles in both ovaries of control and treated animals were analysed. The numbers of follicles analyzed for each quantification were as follows: controls (early preantral n=18, preantral n=71; antral n=64), ABT-898 (early preantral n=14, preantral n=58; antral n=68).

### 5.3 Assays

Blood samples were collected three times per week for 6-8 weeks pre-treatment and during the study period in ABT-898 treated marmosets, for the determination of plasma progesterone and ABT-898 levels. Progesterone concentrations were determined by radioimmunoassay (RIA) using antiserum R31/12 as described previously (Smith et al., 1990).

#### 5.3.1.1 ABT-898 ELISA

The anti-ABT-898 polyclonal antibody 983-1c was prepared by Abbott Laboratories. It was generated using Invitrogen Custom Peptide/Antibody Services (Invitrogen, Carlsbad CA). A cysteine appended synthetic peptide analogue of ABT-898 (Ac-Gly-val-(d)alloile-ser-gln-ile-arg-pro-cys-amide) was coupled to keyhole limpet hemocyanin (KLH) and used to immunize rabbits. The resulting ABT-898-cross reactive antisera were affinity purified and serum # 983-1c was utilized for development of an ABT-898 ELISA by Abbott Laboratories. Plasma concentrations of ABT-898 were determined using a competition ELISA carried out by Mr Ian Swanston. A biotinylated peptide analogue of ABT-898 was diluted in blocking buffer (0.1% BSA, 0.05% Tween in PBS (0.1M phosphate, 0.15M NaCl, pH 6.9-7.2) and added to neutravidin pre-blocked plates (Pierce Biotech, Rockford, IL ) at room temperature for 1 h. Wells were washed three times with a wash buffer containing 0.05% Tween-20 in PBS. ABT-898 standards diluted in serum or test serum samples were then added to appropriate wells followed by the immediate addition of anti-898 Ab, 983-1c. The plates were then incubated for 30min. After incubation, wells were

washed three times with wash buffer. A goat anti-rabbit peroxidase secondary antibody solution was added to the wells and plates were incubated at room temperature for 30min. Next, 1-step ultra TMB solution (Pierce Biotech, Rockford, IL) was added for 15min and the reaction was stopped by addition of 2N Sulphuric Acid. The OD was determined at 450nm. Assay sensitivity was 2ng/well, and interassay variation based on low-, medium-, and high-quality controls were less than 10%. Concentration vs. time curves were constructed from ELISA-generated ABT-898 values obtained from individual animals. The pharmacokinetic parameter estimates were determined by fitting the serum concentration vs. time profile to a noncompartmental model (WinNonLin, version 2.0, Pharsight Corp., Mountain View, CA).

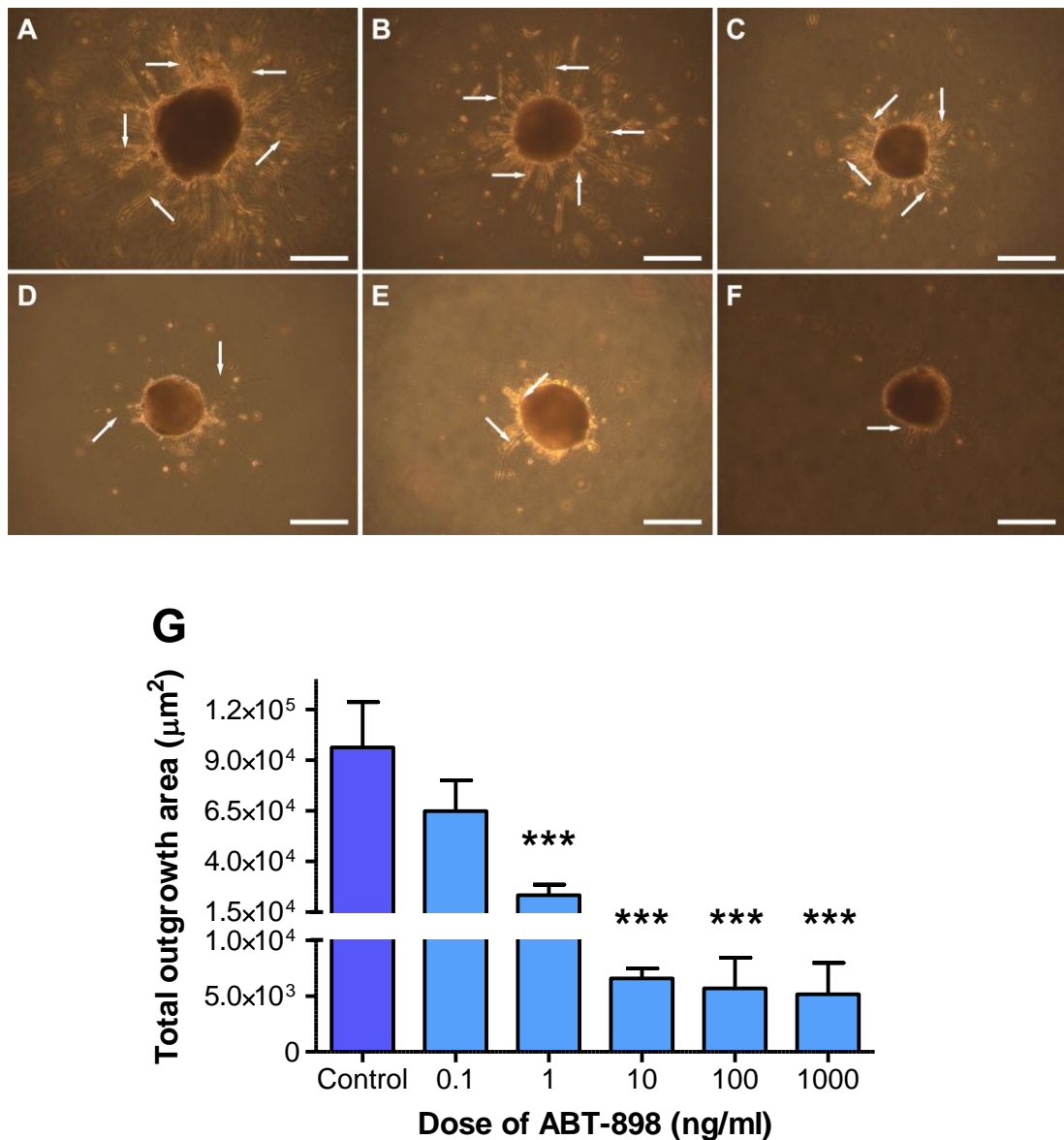
### 5.3.2 Statistical Analyses

Data are presented as mean  $\pm$  SEM and all results were analysed statistically using Graph Pad Prism 5 software (Graph Pad Software). The data for figures 5.4, 5.5, 5.6 and 5.7 were analysed by two-tailed t-tests with a 95% confidence interval to compare the day 0 data with the day 6 data and statistical significance is indicated by \*. For the progesterone results (Fig 5.3), the post-treatment period data was subjected to statistical analysis and this was based on the duration of the normal luteal phase. This was achieved by comparing the area under the curve for pre-treatment and post-treatment cycles. The rest of the results in this chapter were analysed by means of a One-way ANOVA with a Bonferroni post-hoc test performed if the 95% confidence interval was reached to determine significance between all of the data groups. P values  $<0.05$  were accepted as statistically significant.

## Results

### 5.3.3 Effect of ABT-898 on follicular angiogenesis *in vitro*

To investigate the effect of ABT-898 on follicular angiogenesis *in vitro*, preantral and early antral rat follicles (n=12 per treatment) were cultured in serum-free medium treated with 0.1, 1, 10, 100 and 1000ng/ml ABT-898 for 6 days. Treatment with ABT-898 resulted in a significant inhibition of follicular angiogenesis, in a dose dependent manner, (Figure 5.1) ( $P<0.001$ ), showing that ABT-898 is able to inhibit angiogenesis in whole ovarian follicles, *in vitro*.



### Figure 5.1 Effect of ABT-898 on angiogenesis *in vitro*

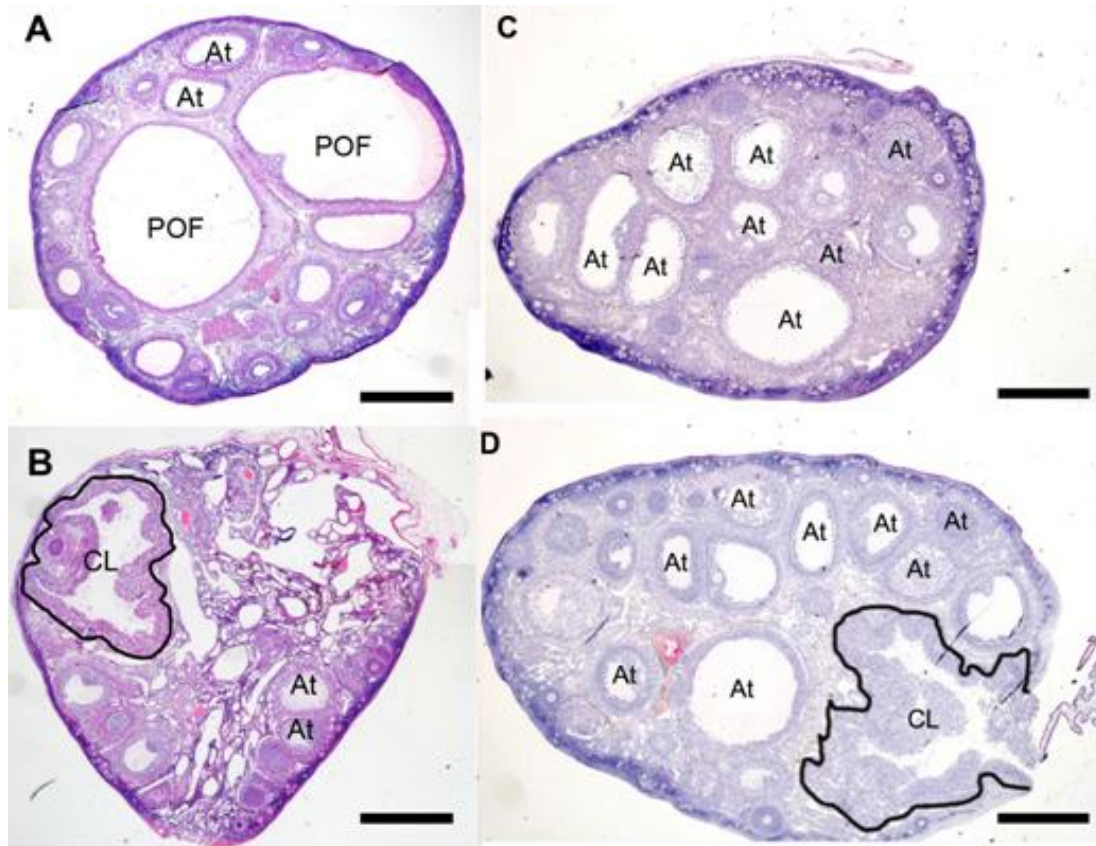
To determine the effect of ABT-898 on angiogenesis isolated preantral and early antral follicle ( $n=6$  per treatment) were with varying doses of ABT-898. At the end of the culture period the area of the angiogenic outgrowths was measured using Image Pro-Plus software. Representative images show endothelial outgrowths from a control follicle (A) and follicles after treatment with ABT-898 at 0.1ng/ml (B), 1ng/ml (C), 10ng/ml (D), 100ng/ml (E) or 1000ng/ml (F). The results were analysed statistically using a One-way ANOVA with a Bonferroni post-hoc test to compare the ABT-898 treated follicles to the control follicles. Treatment with ABT-898 resulted in a significant dose-dependent decrease ( $***P<0.001$ ) in outgrowth area (G). Arrows indicate the endothelial outgrowths. Scale bars represent 200μm. Experiments were repeated in triplicate.

### 5.3.4 Treatment with ABT-898 throughout the follicular phase

Prior to the *in vivo* study, the concentration of ABT-898 in plasma following a single injection of 2.5mg/kg was measured and it found to be detectable for up to 8h. At 15min after injection, plasma concentrations of ABT-898 reached a mean value of 26,500 ng/ml, falling to 1,747 ng/ml by 4h. The last time-point at which ABT-898 was still detectable, average 147ng/ml, was at 8h post-injection but was below detection limit (5ng/ml) after 12 hours. The half life was 0.83h with clearance parameter of 0.039. This compares with values of 1.2h and 0.12 respectively in cynomolgus monkeys (Abbott Laboratories, unpublished). Therefore, for the treatment schedule, it was decided to administer ABT-898 twice daily to ensure that levels remained within the effective range.

### 5.3.5 Effect of ABT-898 on ovulation and progesterone production

In ovaries (n=10) from control marmosets (n=5) the predominant structures on day 10 were preovulatory follicles (n=6; mean per ovary=0.6) and newly formed corpora lutea (n=9; mean per ovary=0.9) (Figure 5.2A-B). Ovaries from ABT-898 treated marmosets (n=5) were also dominated by pre-ovulatory follicles (n=7; mean per ovary=0.7) or newly formed corpora lutea (n=9; mean per ovary=0.9) (Figure 5.2C-D), showing that treatment with ABT-898 did not suppress follicle selection or ovulation. In accordance with this data, paired ovarian weights of ABT-898 treated marmosets at day 10 (260mg  $\pm$ 49) were not significantly different from controls (223mg  $\pm$ 43). There was also no difference in the size of the preovulatory follicles or the corpora lutea between the control ovaries and the ABT-898 treated ovaries.

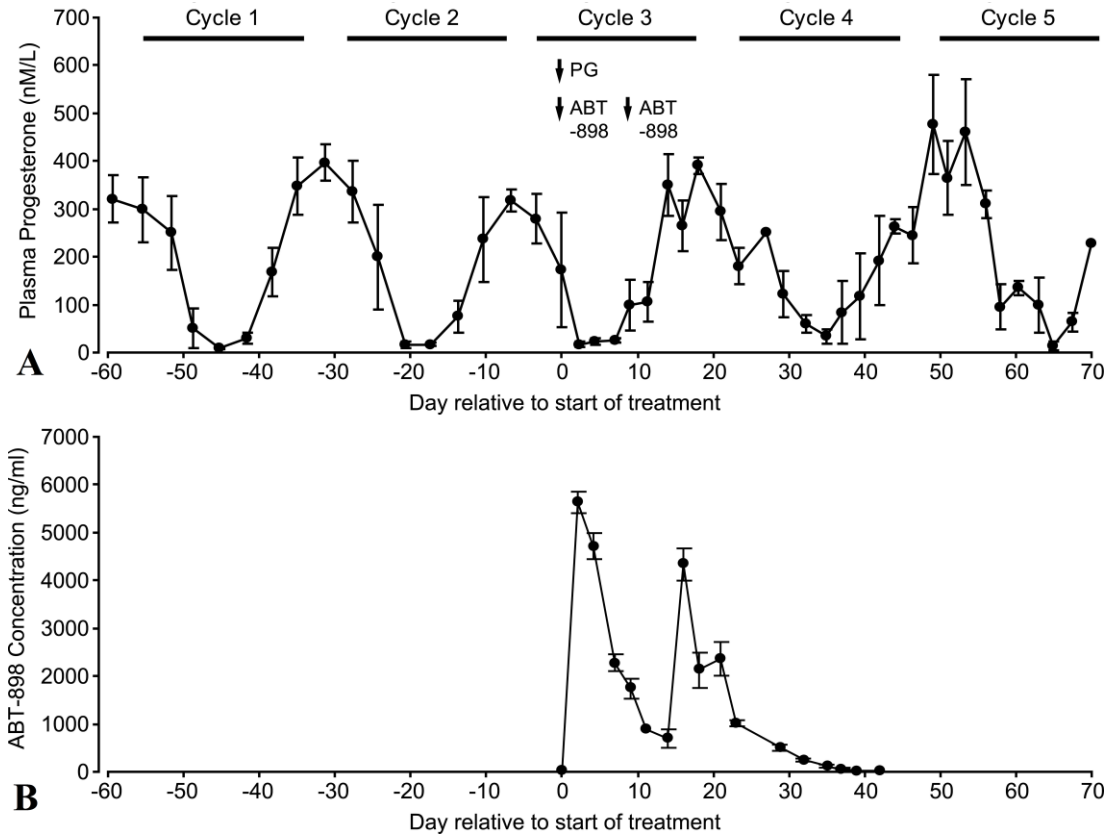


**Figure 5.2 Effect of ABT-898 on follicular development and ovulation**

To investigate the effects of a TSP-1-mimetic peptide, ABT-898, *in vivo*, marmoset monkeys were treated with ABT-898 or a vehicle control. At the end of the treatment the ovaries were removed and fixed, sectioned and stained for histological analysis. Representative images show haematoxylin and eosin staining in marmoset ovaries 10 days after treatment with vehicle control (A, B) or with ABT-898 (C, D). Note the main structures in the control ovaries were preovulatory follicles (POF) and corpora lutea (CL (outlined)), together with large antral follicles, the majority of which are healthy. The main structures in the treated ovaries were also POFs and CLs but the majority of the remaining large follicles were atretic (At). Both ovaries from 5 control and 5 treated marmosets were analysed and 3 slides at different points through each ovary were counted. Scale bars represent 1000µm.

It has previously been shown that treatment with angiogenesis inhibitors can block ovulation and inhibit progesterone secretion (Taylor et al., 2007). Long-term treatment with ABT-898 depot resulted in maintenance of high levels of the peptide in the circulation, but this had no effect on the timing or magnitude of the normal rise in plasma progesterone levels, which occurred around 10 days after the initiation of treatment. The second injection also failed to affect the length of the luteal phase or peak progesterone concentrations (Figure 5.3).



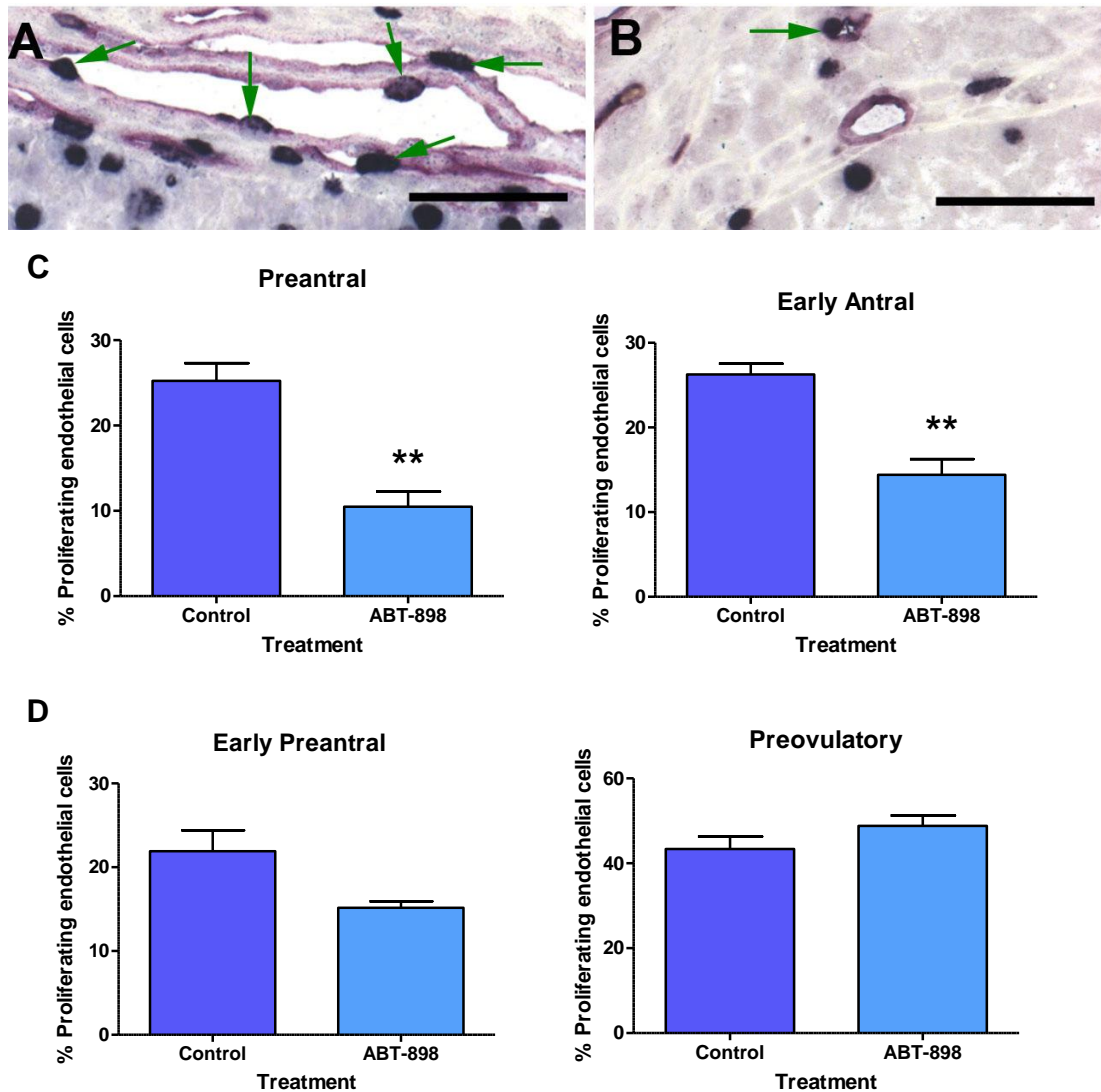


**Figure 5.3 Effect of ABT-898 depot on ovulatory cycles**

Marmosets were treated with a vehicle control or ABT-898 for 10 days and blood samples were taken from these marmosets every three days for three cycles prior to treatment and for two cycles during and then post-treatment to allow hormonal profiling. The concentration of plasma progesterone (PG) during three pre-treatment control cycles is shown (A). At the third cycle, PG injection is used to induce luteolysis simultaneous to the first injection of ABT-898 depot (arrow). Ovulation occurred at the normal time. A second depot injection of ABT-898 given at the resulting early luteal phase failed to effect progesterone concentrations or the timing of the next ovulation. Data was analysed by comparing the area under the curve for the pre- and post-treatment cycles. The plasma profiles of ABT-898 following the two injections are shown (B). Note the high levels of peptide maintained throughout the study period.

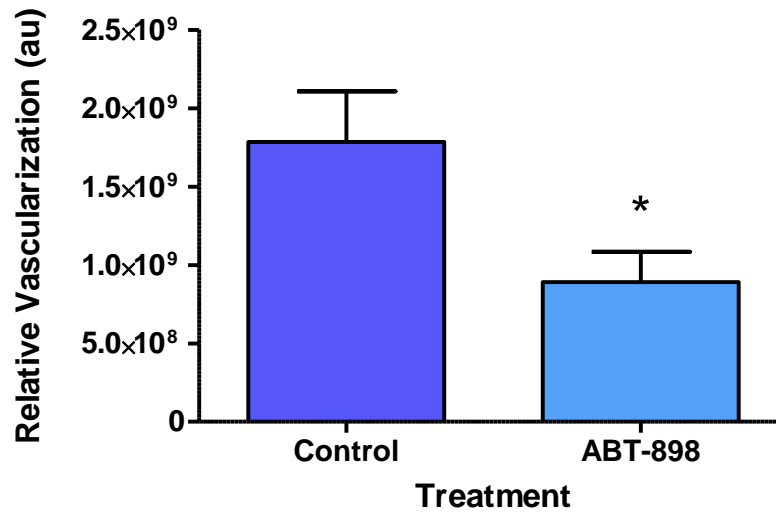
### **5.3.6 Effect of ABT-898 on angiogenesis and granulosa cell proliferation**

As the ovary is dynamic and undergoes intense angiogenesis during both folliculogenesis and corpus luteum formation, there are normally a high number of proliferating endothelial cells within the thecal layer of developing follicles and in the corpus luteum. Although treatment with ABT-898 failed to block the emergence of the dominant follicles or ovulation, there were significant changes in other classes of developing follicles within these ovaries. ABT-898 treatment resulted in a significant reduction in follicular angiogenesis in preantral and early antral follicles, as determined by the reduction in the proportion of proliferating endothelial cells (shown by dual staining with BrdU and CD31) (Figure 5.4A) compared with controls (Figure 5.4B). The inhibition of angiogenesis was found to be significant in late preantral and antral follicles (Figure 5.4C), but not in early preantral or preovulatory follicles (Figure 5.4D). Quantification of the area of CD31 staining in the theca showed a significant reduction ( $P<0.05$ ) in vascular area after treatment with ABT-898 (Figure 5.5).



**Figure 5.4 Effect of ABT-898 on endothelial cell proliferation**

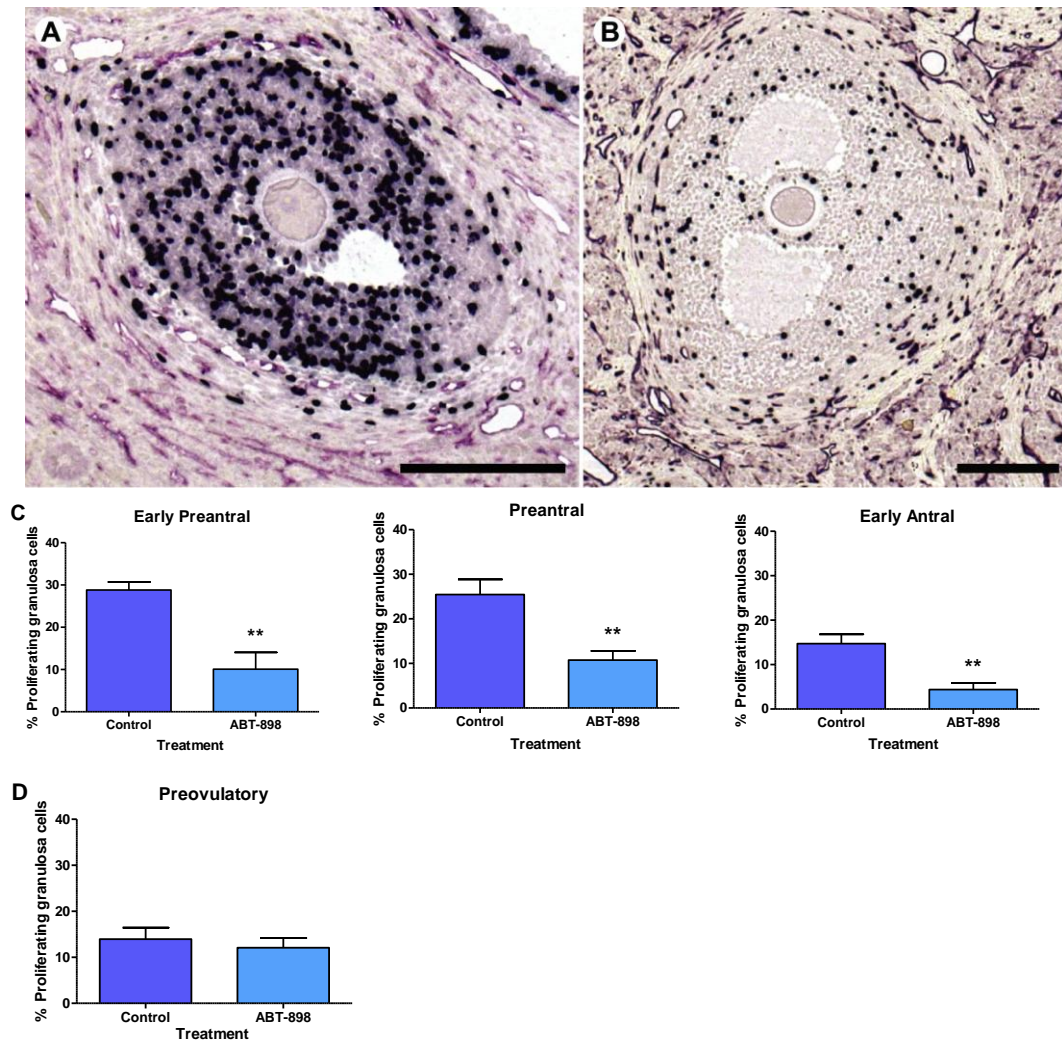
To investigate the effects of ABT-898 on *in vivo* angiogenesis marmoset monkeys were treated with ABT-898 or a vehicle control. At the end of the treatment the ovaries were removed and fixed, sectioned and stained for histological analysis. Representative images show dual staining for BrdU and CD31 in marmoset ovaries to identify proliferating endothelial cells (arrows), in control (A) and treated (B) follicles. The results were analysed statistically using two-tailed *t*-tests to compare the different treatment groups. There was a significant decrease in follicular angiogenesis after treatment with ABT-898, when compared to the control, in both preantral and early antral follicles (\*\* $P < 0.01$ ) (C). There was no change in follicular angiogenesis after treatment with ABT-898 in both early preantral and preovulatory follicles (D). Both ovaries from 5 control and 5 treated marmosets were analysed and 3 slides at different points through each ovary were counted. Scale bars represent 50  $\mu$ m.



**Figure 5.5 Effect of ABT-898 on relative vascularisation**

*To investigate the effects of ABT-898 on in vivo angiogenesis marmoset monkeys were treated with ABT-898 or a vehicle control. At the end of the treatment the ovaries were removed and fixed, sectioned and stained for histological analysis. The level of vascularisation in the thecal layer of follicles in both the control and treated ovaries were quantified and the results were analysed statistically using two-tailed t-tests to compare the ABT-898 treated ovaries to the control ovaries. There was a significant decrease in the relative vascular investment after ABT-898 treatment in all stages of follicle development (\* $P < 0.05$ ). The left and right ovaries from 5 control and 5 treated marmosets were analysed and 3 slides at different points through each ovary were counted.*

In addition, treatment with ABT-898 resulted in a significant reduction in granulosa cell proliferation from ovaries of control (Figure 5.6A) and ABT-898 treated marmosets (Figure 5.6B). The inhibition of granulosa cell proliferation was found to be significant in early preantral, late preantral and early antral follicles when compared with control ovaries (Figure 5.6C), but not in dominant, preovulatory follicles (Figure 5.6D).



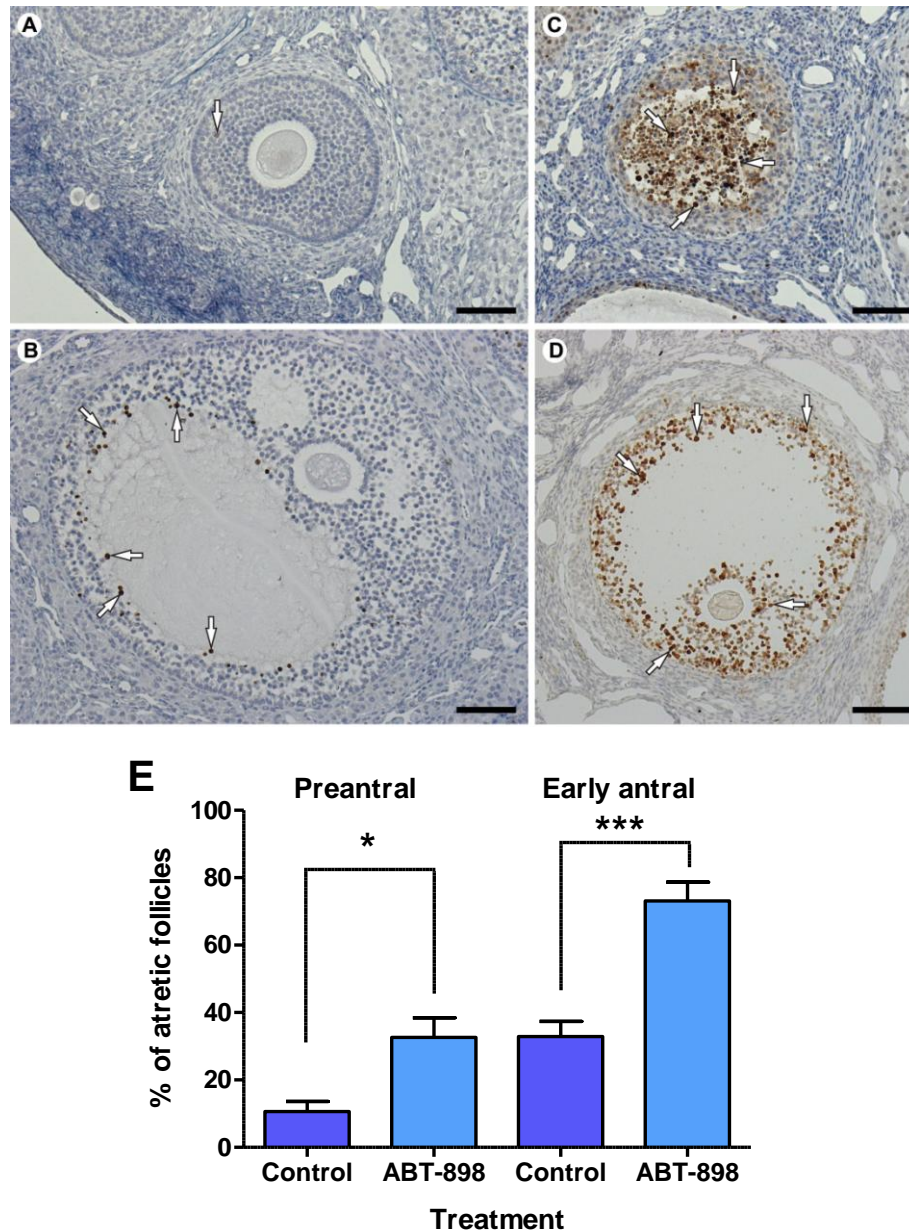
**Figure 5.6 Effect of ABT-898 on granulosa cell proliferation**

To investigate the effects of ABT-898 on cellular proliferation marmoset monkeys were treated with ABT-898 or a vehicle control. At the end of the treatment they were injected with BrdU and the ovaries were removed and fixed, sectioned and stained for histological analysis. Representative images show staining for BrdU in marmoset ovaries to identify proliferating granulosa cells, in control (A) and treated (B) follicles. Early antral follicles are shown from both control (A) and ABT-898 treated ovaries (B). The results were analysed statistically using two-tailed t-tests to compare the ABT-898 treated ovaries to the control ovaries. There was a significant decrease in granulosa cell proliferation after treatment with ABT-898 in early preantral, preantral and early antral follicles, when compared to their respective control (\*\* $P < 0.01$ ) (C). There was no change in granulosa cell proliferation after treatment with ABT-898 in preovulatory follicles (D). Both ovaries from 5 control and 5 treated marmosets were analysed and 3 slides at different points through each ovary were counted. Scale bars represent 200 $\mu$ m.

### **5.3.7 Effect of ABT-898 on follicular atresia**

A proportion of developing follicles undergo atresia during normal follicular development (Figure 5.7A and B). Treatment with ABT-898 resulted in a significant increase in the level of staining for activated caspase-3 (Figure 5.7C and D) in preantral and early antral follicles. There was no difference in the level of activated caspase-3 staining in early preantral and preovulatory follicles (results not shown). This was indicative of a significantly higher number of atretic follicles in the ABT-898 treated ovaries, compared with the control ovaries (Figure 5.7E).





**Figure 5.7 Effect of ABT-898 on follicular atresia**

To determine the effect of ABT-898 on follicular atresia marmoset monkeys were treated with ABT-898 or a vehicle control. At the end of the treatment the ovaries were removed and fixed, sectioned and stained for histological analysis and then analysed for the expression of apoptotic markers. Representative preantral and early antral follicles from control (A and B) and ABT-898 treated marmoset ovaries (C and D) are shown. The results were analysed statistically using a two-tailed t-test to compare the ABT-898 treated ovaries to their respective control ovaries. There was no difference in the level of activated caspase-3 staining in early preantral and preovulatory follicles (results not shown). Treatment with ABT-898 resulted in a significant difference in % of atretic follicles between control and ABT-898 treated ovaries in pre- and early antral follicles (E). \* indicates  $P < 0.05$ , \*\*\* indicates  $P < 0.001$ , in comparison with the respective control value. Both ovaries from 5 control and 5 treated marmosets were analysed and 3 slides at different points through each ovary were counted. Arrows indicate staining for activated caspase-3 (brown). Scale bars represent 100 $\mu$ m.

## 5.4 Discussion

This chapter has shown for the first time the effects of the thrombospondin-1 mimetic peptide ABT-898 on follicular angiogenesis and development. Treatment with ABT-898 resulted in the suppression of follicular angiogenesis, a reduction in endothelial cell proliferation at the pre- and early antral stages of follicular development and the suppression of granulosa cell proliferation at early preantral, preantral and early antral stages of follicular development. In addition to the inhibition of angiogenesis, ABT-898 treatment also resulted in a significant increase in the number of preantral and early antral follicles undergoing apoptosis. However, follicle selection and ovulation were not inhibited by treatment, suggesting that the effects of ABT-898 are stage-specific.

Angiogenesis is necessary for normal follicular development and is stimulated in preantral follicles by the pro-angiogenic factor VEGF (Wulff et al., 2002, Ferrara, 2004, Tamanini and De Ambrogi, 2004). The previous chapter showed that TSP-1 is able to inhibit follicular angiogenesis *in vitro* and the present study has demonstrated that the peptide ABT-898 inhibits follicular angiogenesis *in vitro* and *in vivo*. Treatment with ABT-898 resulted in specific changes in cellular proliferation within developing follicles. Endothelial cell proliferation in the thecal layer of preantral and early antral follicles was inhibited and there was reduced overall vascularisation in the theca of ABT-898 treated marmosets. Treatment with ABT-898 was also associated with the suppression of granulosa cell proliferation in developing follicles. TSP-1 is expressed most highly in follicles at the pre- and early antral stages of development and expression decreases as follicles progress to the preovulatory stage



(Petrik et al., 2002). As treatment with ABT-898 both *in vitro* and *in vivo* leads to the inhibition of follicular angiogenesis, and as TSP expression declines with increased follicular angiogenesis, it is likely that TSP acts to restrict angiogenesis in follicles during early follicular development. It has previously been shown that TSP-1 and VEGF are expressed in an inverse pattern (Greenaway et al., 2005) and as VEGF expression increases as folliculogenesis progresses it is likely that the greater levels of VEGF expressed in antral and preovulatory follicles would counteract the inhibitory effect of TSP-1 on follicular angiogenesis, meaning that preantral and early antral follicles would be more susceptible to the inhibition of angiogenesis caused by TSP-1.

Regulation of the inhibitory effects of TSP-1 on follicular angiogenesis is likely to be complex. It has been shown that TSP-1 signals through both the CD36 and CD47 receptors which induce different responses, as they interact with different downstream molecules. TSP-1 signalling via CD36 inhibits angiogenesis and VEGFR2 which results in the blockade of the pro-angiogenic activity of VEGF (Dawson et al., 1997, Zhang et al., 2009). Follicles at the pre- and early antral stages of development also have lower expression of VEGF (Ferrara, 2004, Fraser et al., 2005a) so the balance of the numerous other factors involved in angiogenesis becomes more significant and ABT-898 is able to reduce follicular angiogenesis. However, TSP-1 signalling via CD47 has been shown to up regulate the expression of matrix metalloproteinase (MMP)-9 (Qian et al., 1997, Donmez et al., 2009). MMPs play an important role in angiogenesis as they degrade basement membrane collagens, thus allowing endothelial cells to migrate and proliferate (Bendeck, 2004).

In addition, MMP-9 can stimulate the expression of VEGF (Donmez et al., 2009), suggesting that TSP-1 could increase the expression of pro-angiogenic factors despite being an endogenous angiogenesis inhibitor. Moreover, VEGF has been shown to inhibit the expression of TSP-1 and as the expression of VEGF increases as follicle development progresses it is likely that inhibitory actions of ABT-898 are overcome in dominant follicles due to higher expression of VEGF. It is not surprising that TSP-1 has both stimulatory and inhibitory effects on angiogenesis as it interacts with numerous receptors and proteases, however, in the ovary all the evidence so far suggests that it has an inhibitory role. Importantly, it has recently been shown that the levels of TSP-1 expression are reduced in women with PCOS (Tan et al., 2009), supporting the evidence for TSP-1 inhibiting angiogenesis in the ovary.

Expression of TSP-1 has been shown to be up-regulated in follicles undergoing atresia (Thomas et al., 2008) and the previous chapter showed that TSP-1 treatment *in vitro* induces follicular apoptosis. Therefore it was hypothesised that treatment with ABT-898 *in vivo* would have a similar effect. This chapter has demonstrated for the first time that treatment with ABT-898 induces follicular atresia in a stage-specific manner in the ovary and this, along with the *in vitro* data (Garside et al., 2010), suggests that TSP may play an important role in the clearance of non-dominant follicles within the ovary. TSP-1 has been shown to inhibit the proliferation and migration of endothelial cells (Lawler, 2002, Folkman, 2004) and the resulting decrease in angiogenesis may be at least partly responsible for the induction of follicular atresia. In addition, treatment with TSP-1 has been shown to

directly induce apoptosis of endothelial cells (Guo et al., 1997, Jimenez et al., 2000, Nor et al., 2000). Since the TSP-1 receptor CD36 is expressed on granulosa cells (Dawson et al., 1997, Petrik et al., 2002, Thomas et al., 2008) and TSP-1 has been shown to induce granulosa cell apoptosis directly (Garside et al., 2010), it is also likely that TSP-1 is able to induce follicular atresia *in vivo* independently of angiogenesis inhibition. Here we have demonstrated that treatment with ABT-898 reduced both granulosa and endothelial cell proliferation, suggesting a dual effect of TSP-1 to promote follicular atresia. It is possible that the effect on granulosa cell death could be due to a cytotoxic effect of ABT-898. However, toxicology studies performed by Abbott Laboratories (unpublished observations) both *in vitro* and *in vivo* have shown that ABT-898 does not induce mutagenesis or clastogenesis in a variety of cells, including Chinese hamster ovary cells. In this study we have shown that treatment with ABT-898 induces follicular atresia but that a cohort of healthy follicles is present in all of the treated ovaries. If this effect was due to cytotoxicity of the compound all follicles would be affected equally and this is not the case, suggesting that the apoptosis seen is a manifestation of ABT-898 mimicking the physiological effect of TSP.

Previous studies have investigated the effect of several anti-angiogenic agents in the marmoset and treatment with Aflibercept, a VEGF inhibitor, at specific stages of the cycle has been shown to inhibit follicle selection, prevent ovulation, induce rapid luteolysis and suppress progesterone secretion (Wulff et al., 2002, Fraser et al., 2006). It was hypothesised that treatment with ABT-898 may have similar effects due to its anti-angiogenic activity and this was investigated in non-terminal studies.

Marmosets treated with ABT-898 in a long-acting depot formulation showed no inhibition of progesterone production and went on to cycle normally, producing preovulatory follicles and corpora lutea that were of comparable size and frequency to those seen in control animals. Progesterone secretion continued as normal after treatment with ABT-898, showing that ABT-898 does not induce luteolysis nor inhibit ovulation, unlike Aflibercept. TSP-1 has been shown to act, at least in part, through inhibition of VEGF (Greenaway et al., 2007) but it appears that this partial inhibition of VEGF action is not sufficient to inhibit progesterone production and ovulation. To assess function of the preovulatory follicles apparently unaffected by treatment with ABT-898 it would have been informative to determine peak levels of plasma estradiol. However, detecting subtle differences in estradiol in marmosets may be confounded by high levels of circulating estrone (unpublished observations). To overcome this, the effects of ABT-898 depot treatment on estradiol secretion were investigated in the stump-tailed macaque. No change in serum estradiol was detected (H.M. Fraser, unpublished observations).

The depot treatment was designed to investigate the immediate effects on major cycling events, such as ovulation and luteal function, as it has been shown that inhibition of angiogenesis can block ovulation (Taylor et al., 2007). As ABT-898 treatment targets follicles at the preantral and early antral stages of development it is likely that any effects on ovulation and progesterone secretion would not be seen in the treatment cycle, but future cycles, as that would be when the targeted follicles would reach ovulation. In this study blood samples were collected for three months after treatment but as this is only 2 cycles post-treatment it is possible that effects on

ovulation and progesterone secretion would be seen later on. On the other hand, it is possible that ABT-898 targets follicles that are not destined to ovulate and this would explain why ovulation still occurred as normal, both in the treatment cycle and in the post-treatment cycles. It has previously been shown that treatment with Aflibercept blocked ovulation with immediate effect (Taylor et al., 2007) and this is likely to be due to the absolute requirement for VEGF for adequate follicular angiogenesis. TSP also inhibits angiogenesis but as TSP targets preantral and early antral follicles, angiogenesis still occurs in more developed follicles in the presence of TSP, thus allowing sufficient follicle developments during the treatment cycle to allow ovulation to occur.

This chapter has shown for the first time that treatment of marmosets with a potent TSP-1 mimetic peptide, ABT-898, results in the reduction of angiogenesis in preantral and early antral follicles. In addition we have shown that ABT-898 is able to induce follicular atresia, *in vivo*. Angiogenesis is an important process in many pathological conditions and the regulation of angiogenesis is complex due to the interaction of the many pro- and anti-angiogenic factors involved in this process. Therefore, research that helps elucidate the role of these factors in angiogenesis and the ability to manipulate angiogenesis *in vivo* is of clinical importance. Angiogenesis is increased in PCOS and atresia of antral follicles is reduced (Agrawal et al., 1998a, Franks et al., 2000) so TSP-1 could be a potential treatment for this condition as it could have a dual role in targeting the increased angiogenesis and inducing atresia of accumulated antral follicles without affecting preovulatory follicle development and

ovulation. To further investigate the clinical possibilities of treatment of PCOS with TSP-1 studies in an animal model of PCOS would be required.

There are many animal models available for the study of PCOS that have been developed over several decades and they provide insight into the pathophysiology of PCOS. The animals most commonly used for PCOS models are rodents, sheep and non-human primates and whilst they have all contributed to the understanding of the pathophysiology of PCOS, several of these models lack the traits required to be classed as PCOS models (Abbott et al., 2007, Franks, 2009). Models based on foetal programming appear to be the most representative of human PCOS and also exhibit the metabolic defects that are common in women with PCOS (Abbott DH et al., 2006). Models of foetal programming use various developmental windows to expose foetuses to high levels of androgen and whilst they all result in PCOS, there are phenotypic differences in the presentation of PCOS in early treated and late treated animals (Vendola et al., 1998, West et al., 2001, Abbott et al., 2002, Abbott DH et al., 2006).

The rhesus monkey is commonly used for non-human primate PCOS models and many studies have shown that the prenatally androgenised rhesus monkey develops PCOS-like abnormalities including multi-follicular ovaries, hyperandrogenism, altered LH production and metabolic abnormalities such as insulin resistance (Steiner et al., 1976, Abbott et al., 1998, Abbott et al., 2002, Eisner et al., 2002, Eisner et al., 2003, Dumesic et al., 2005, Franks, 2009), suggesting that this model reliably produced traits that are closely related to PCOS in women.

More recent experiments with prenatally androgenised female sheep (Robinson et al., 1999, West et al., 2001) support the findings in the rhesus monkey that prenatal androgen exposure to high levels of androgen at key windows of development results in a PCOS-like phenotype. Studies in prenatally androgenised rodents also support these findings (Sullivan and Moenter, 2004, Foecking et al., 2005), which indicates that the effects of prenatal exposure to high levels of androgen are concurrent across the different species. The various animal models have helped to improve the understanding of the pathophysiology of PCOS but no one model can provide all the answers as each have their advantages and disadvantages. Therefore, to investigate the potential effects of TSP-1 in PCOS it would be most informative to use a variety of animal models. There are many other factors that play an important role in the angiogenic cascade so the role of Ang-1 in ovarian angiogenesis will be investigated using the *in vitro* angiogenesis assay.

## **Chapter 6 Angiopoietins Promote Angiogenesis and Follicle Survival**



## **6 Chapter 6 Angiopoietins Promote Angiogenesis and Follicle Survival**

### **6.1 Introduction**

Although VEGF is the key factor involved in stimulating angiogenesis, the angiopoietins are considered to be the most important class of proteins that act in concert with VEGF to produce and maintain stable blood vessels (Maisonpierre et al., 1997). The angiopoietins are cytokine glycoproteins which act in a competitive manner via the tyrosine kinase receptor Tie-2 (Maisonpierre et al., 1997). Four angiopoietins have been identified so far, with the best characterised being angiopoietin (Ang)-1 and Ang-2. Previous studies have shown that Ang-1 and Tie-2 knockout mice exhibit embryonic lethality and have a high degree of blood vessel immaturity and disorganisation (Dumont et al., 1994, Sato et al., 1995, Suri et al., 1996, Jones et al., 2001), showing that the angiopoietins are essential for vascular development and remodelling. It has been shown that Ang-1 plays a role in supporting and stabilising newly developed blood vessels (Thurston et al., 2005) and that it is able to induce angiogenesis in endothelial cells, *in vitro* (Koblizek et al., 1998). Ang-2 has considerable sequence homology to Ang-1 but can both aid the development of new blood vessels and aid vascular regression, dependent on the circumstances, through antagonism of the Tie-2 receptor (Maisonpierre et al., 1997, Asahara et al., 1998, Witzenbichler et al., 1998).

Ang-1 and Tie-2 are expressed in the ovarian follicle in the rodent, bovine and primate; however the expression pattern of this ligand and its receptor varies across

species. In the primate, specifically the marmoset, Tie-2 expression is localised in the vasculature of the thecal layer of antral follicles, but is also apparent in the granulosa cells of primordial, early preantral and preantral follicles as determined by *in situ* hybridisation (Wulff et al., 2001a). The bovine follicle expresses mRNA for Ang-1 and Tie-2 in the thecal layer; however the granulosa cells express mRNA for the ligand only (Hayashi et al., 2003). Finally, it has been shown by IHC in the rat that Ang-1 expression is confined to the thecal endothelium of follicles; that it increases as the follicle develops, and is not present at any stage in the granulosa cells (Abramovich et al., 2009).

Treatment of rat ovaries *in vivo* with an antibody to Ang-1 has been shown to promote follicular atresia (Parborell et al., 2008), suggesting that Ang-1 may have an anti-apoptotic effect on granulosa cells. Since Tie-2 receptors have not been previously localized in rat granulosa cells, it was concluded that Ang-1 promoted granulosa cell survival in a paracrine manner mediated via the Tie-2 receptor in the endothelial cells of the thecal layer. However, the presence of Tie-2 expression in granulosa cells of primordial, primary and preantral follicles in the marmoset ovary (Wulff et al., 2001a), and in antral follicles in the horse and sheep (Ellenberger et al., 2009, Chowdhury et al., 2010), suggests that Ang-1 may have an extravascular role during follicular development by acting directly on granulosa cells.

It has previously been shown that phosphorylation of Tie-2 by Ang-1 activates Akt (Augustin et al., 2009), which then activates pro-survival pathways and suppresses mediators of apoptotic pathways. There is increasing evidence that important

interactions occur between the PI-3 kinase/Akt pathway and members of the MAPK family (Papapetropoulos et al., 2000, Gratton et al., 2001, Harfouche et al., 2003, DeBusk et al., 2004). The best characterized members of the MAPKs include the extracellular signal-regulated kinases (ERKs) and the p38 kinases (Robinson and Cobb, 1997), which have anti- and pro-apoptotic functions, respectively. In addition, Akt signalling leads to inactivation of the forkhead transcription factor, FOXO1 (Augustin et al., 2009), which blocks cell division and promotes apoptosis and has been shown to be expressed on granulosa cells (Bastie et al., 2005). It has also been reported that VEGF is able to activate the Tie-2 receptor in HUVECS independently of Ang-1 binding via cleavage of Tie-1 (Singh et al., 2009). If this occurs in granulosa cells it may have important implications for follicular development.

In this chapter, the effects of Ang-1 on follicular angiogenesis were investigated using the *in vitro* angiogenesis assay described in Chapter 3. In addition, as current literature is unclear regarding the localisation of Tie-2, expression of Tie-2 receptor mRNA and protein in the rat ovary was localized to elucidate the cell types involved in Ang-1 action. Finally, the signalling pathway(s) activated by Ang-1 and the functional role of Ang-1 on granulosa cell survival were evaluated *in vitro*.

## 6.2 Materials and Methods

### 6.2.1 Follicle Culture

For all experiments, preantral/early antral follicles were cultured individually in flat-bottomed 12-well plates, embedded in the centre of a 20µl droplet of growth-factor reduced Matrigel. The Matrigel provides a three-dimensional extracellular matrix support for follicle growth and allows the vascular outgrowths to develop in three-dimensions, as they would *in vivo*. Follicles were incubated for 6 days in a sterile humidified air atmosphere with 5% CO<sub>2</sub> at 37°C in 600µl serum-free EBM-2 medium supplemented with BSA (0.1%), L-glutamine (3mM), penicillin (100 IU/ml), streptomycin (0.1mg/ml), transferrin (2.5µg/ml), selenium (4ng/ml), insulin (10ng/ml) and L-ascorbic acid, sodium salt (50µg/ml), with culture medium refreshed every 48h.

In order to test the effects of Ang-1, follicles were pooled and randomly placed into groups and cultured in serum-free EBM-2 medium (as described above) in the presence of 0.01 (n=17), 1 (n=18), 10 (n=16) or 100µg/ml (n=18) Ang-1 (Bow-Ang-1, a recombinant Ang-1 tetramer containing an Fc fusion protein (Koblizek et al., 1998, Davis et al., 2003, Huang et al., 2009) (gift from Regeneron Pharmaceuticals, NY, USA). Medium containing an Fc portion of a human immunoglobulin component (gift from Regeneron) at 100µg/ml was used as the control (n=18).

### 6.2.2 Immunohistochemistry

In order to characterise Tie-2 expression during follicular development, ovaries from 4 immature Wistar rats were fixed, embedded and serially sectioned, and tissue

sections (5µm) were placed onto BDH SuperFrost slides. The sections were incubated overnight at 4°C with Tie-2 antibody (1:100 in NGS) and visualisation was achieved by DAB Substrate (see Chapter 2). For the negative controls, the primary antibody was omitted.

## **6.2.3 Granulosa Cell Experiments**

### ***6.2.3.1 Isolation and culture of granulosa cells***

To investigate whether Ang-1 directly affects the health of granulosa cells, isolated granulosa cells were cultured in 0.1ml M199 medium containing  $1 \times 10^4$  cells/well. Following overnight preincubation in a sterile humidified air atmosphere with 5% CO<sub>2</sub> at 37°C, prewarmed 0.1ml M199 medium containing with containing 0, 0.01, 0.1, 1, 10 and 100µg/ml Ang-1 was added to each well for 48h. Treatments were carried out in triplicate and experiments were repeated three times.

### ***6.2.3.2 Caspase-3 and -7 activity assay***

To measure caspase-3 and -7 activities in isolated granulosa cells, the Caspase-Glo 3/7 assay was performed according to the manufacturer's instructions, 48h after treating the cells (see Chapter 2). Treatments were carried out in triplicate and experiments were repeated three times to reduce the possible effects of biological variability. As serum can generate a background caspase activity signal, an additional two wells contained cell culture medium and carrier solution without any cells and TNFα was used as a positive control.

**6.2.3.3 qRT-PCR**

At the end of the culture period, RNA was extracted for analysis, using the RNeasy micro-kit, following the manufacturer's instructions (see Chapter 2). To avoid contamination by genomic DNA, each total RNA sample was treated with deoxyribonuclease, using the DNA-free kit. After RNA extraction, cDNA synthesis was performed using the Vilo cDNA synthesis kit as described in Chapter 2.

Expression of Tie-2 and Ang-2 mRNA in granulosa cells, cultured in the presence or absence of test compounds, was examined using Taqman quantitative PCR as described in Chapter 2. Briefly, a master mix containing 18S primers and probes, 100ng cDNA and primers and probes for the gene of interest were added to each sample. Samples were added in triplicate to a 96 well MicroAmp fast optical reaction PCR plate and analysed using an ABI 7900HT Real-Time PCR System. Expression of analysed genes was normalised to 18S and results are expressed as a percentage change from vehicle treated cells.

**6.2.3.4 RNA interference**

Granulosa cells that were isolated as described above were cultured with GFP-expressing lentivirus to confirm that the lentivirus would be incorporated into this cell type and also to establish the conditions required for efficient transfection and lentivirus that did not express GFP was used as a negative control. The efficiency of transfection was determined by culturing isolated granulosa cells with lentivirus containing miRNA for LacZ at 1 virus/cell. Granulosa cells were isolated and cultured for 48h before being cultured with the LacZ-expressing lentivirus for 8h.

Lentivirus that did not express LacZ was used as a negative control. After 8h the lentivirus was removed and cell were fixed in PBS containing 2% formaldehyde and 0.2% gluteraldehyde for 5min at room temperature. Cells were washed and then stained with X-gal staining solution (23ml 0.5M NaH<sub>2</sub>PO<sub>4</sub>, 77ml 0.5M Na<sub>2</sub>HPO<sub>4</sub>, 1.06g potassium ferrocyanide, 0.82g potassium ferricyanide, 5ml 1% deoxycholate, 5ml 2% NP40, 1ml 1M MgCl<sub>2</sub>, made up to 500ml with dH<sub>2</sub>O; 2ml X-gal added per 100ml staining solution) overnight at 37°C.

RNA knockdown was performed to investigate the importance of Tie-1, Tie-2 and Ang-2 in the angiogenic cascade and follicle health. Oligonucleotides encoding miRNA constructs for Tie-1, Tie-2 and Ang-2 were obtained from Invitrogen and also designed by Pamela Brown and Laura Milne from the in-house Biomolecular Core Facility. The in-house Biomolecular Core Facility then inserted these miRNA constructs into a cloning vector, prior to being inserted into GFP-expressing lentivirus.

Granulosa cells were isolated and cultured for 48h before being cultured with the GFP-expressing lentivirus containing the miRNA construct at 10 viruses/cell, for 8h. After 8h the lentivirus was removed and replaced with fresh medium for 24h. The cells were then treated with Ang-1 for 48h before protein or RNA was extracted from the cells to allow quantification of expression of genes of interest.

### **6.2.4 Western blotting**

Protein was extracted from isolated granulosa cells as described previously (Chapter 2) and were resolved on 4–20% Tris-glycine gels and transferred onto a PVDF-fl membrane. Membranes were blocked for 1h at 25°C in Rockland Near Infra Red blocking buffer and incubated overnight at 4°C in blocking buffer containing rabbit anti-phospho-p42/44 ERK (1:1000), rabbit anti-phospho-p38 (1:500), FOXO1 (1:1000), pTie-2 (1:1000), HIF1 $\alpha$  (1:500), Tie-1 (1:1000), Tie-2 (1:1000) or Ang-2 (1:1000) and  $\beta$ -actin (1:1000). After washing in PBS containing 0.5% Tween, membranes were incubated for 1h at 25°C in the dark in donkey anti goat IgG conjugated to IRDYE 800 and donkey anti rabbit IgG conjugated to Alexa Fluor 680 both at a dilution of 1:5000 in blocking buffer. Membranes were washed and proteins visualised and quantified using an Odyssey Infrared Imaging System. Sample loading was standardised to expression of  $\beta$ -actin.

### **6.2.5 Quantification**

#### ***6.2.5.1 Analysis of Angiogenic Sprouting***

To assess the impact of the treatments on angiogenic sprouting, follicles were examined and photographed directly in the 12-well plates under an inverted light microscope, at x10 magnification, with Axiovision image capturing software on days 0 and 6 of the culture period. The total area of angiogenic sprouting for each follicle was determined using Image Pro Plus software, by drawing an area of interest around the outgrowths (but excluding the follicle) and the sum of the area of all sprouts is measured and calculated by the software. The mean area of angiogenic sprouting for each treatment was then compared to the control.



**6.2.5.2 Analysis of immunohistochemistry**

The expression of Tie-2 was analysed manually at x40 magnification using a tally counter. Expression was determined in the granulosa and thecal layers of early preantral (n=37), preantral (n=49) and early antral (n=75) follicles with the intensity of Tie-2 expression being classified in each follicle using a visual scoring system (1=weak; 2=moderate; 3=strong staining). The percentage of follicles at each intensity was then calculated for each follicle stage and expressed as a mean HSCORE value (Budwit-Novotny DA 1986).

**6.2.5.3 Quantification of X-gal staining and GFP expression**

To assess the efficiency of transfection the total number of cells and total number of X-gal stained cells (blue) were counted in 10 random fields of view and the averages were used to estimate the transfection efficiency using the calculation:

$$\frac{\text{Total number of blue cells}}{\text{Total number of cells}} \times 100 = \% \text{ transfection}$$

To determine the level of GFP-expressing cells the total number of cells and total number of GFP-expressing cells (green) were counted in 10 random fields of view and the averages were used to estimate the percentage of cells expressing GFP.

**6.2.6 Statistical Analyses**

Data are presented as mean  $\pm$  SEM and all results were analysed statistically using Graph Pad Prism 5 software (Graph Pad Software) where appropriate. The results were analysed by means of a One-way ANOVA with a Bonferroni post-hoc test performed if the 95% confidence interval was reached to determine significance

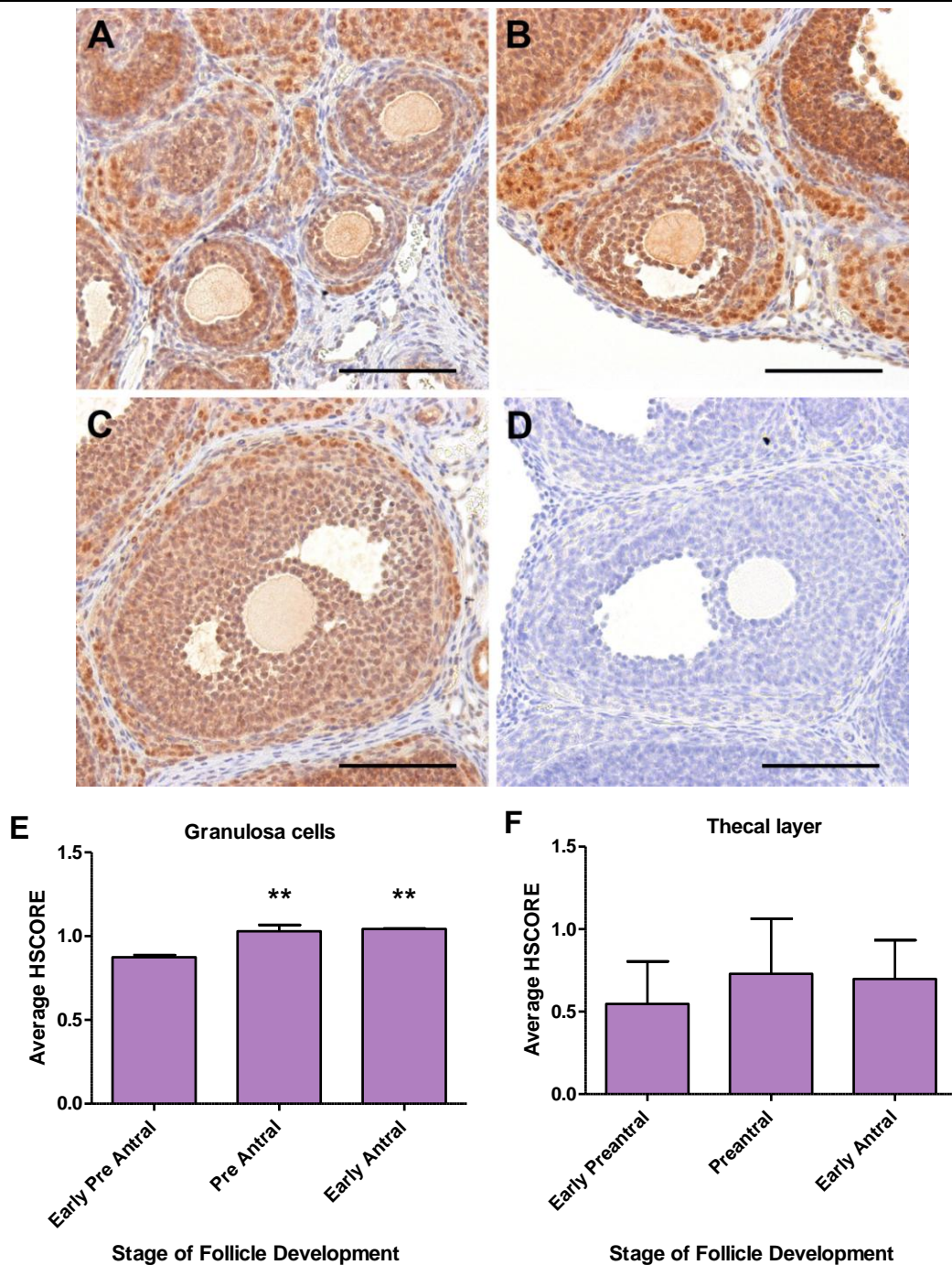
between all of the data groups. P values  $<0.05$  were accepted as statistically significant.

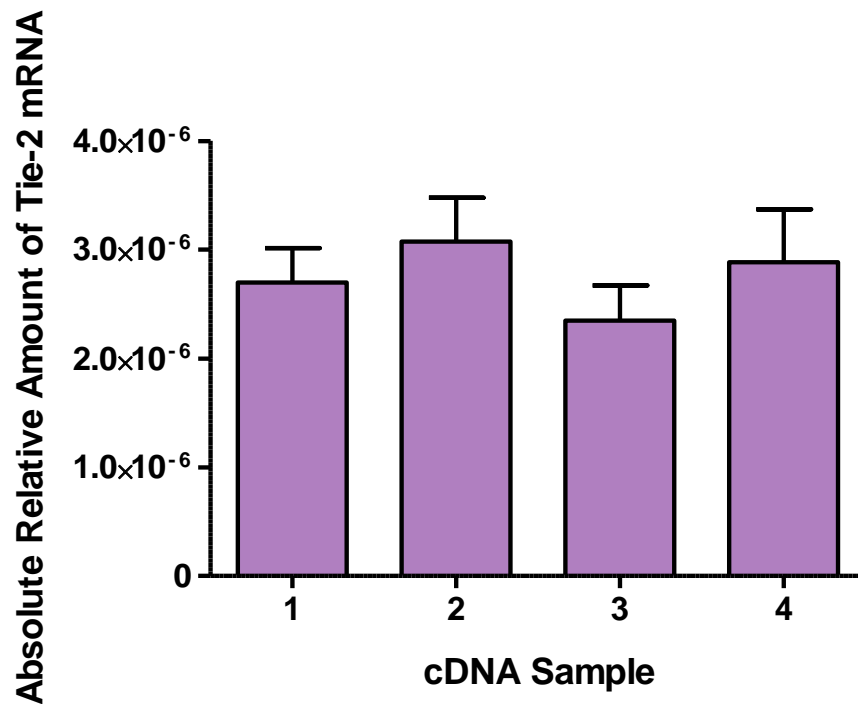
## **6.3 Results**

### **6.3.1 Expression of Tie-2 in the rat ovary**

Tie-2 expression has previously been reported to be expressed exclusively in the thecal endothelium in the rodent ovary (Abramovich et al., 2009). In this chapter, it has been shown for the first time that Tie-2 is expressed both in the granulosa and thecal layers of rat follicles (Figure 6.1A-D). Using immunohistochemistry, expression of Tie-2 protein was found to be higher in granulosa cells of preantral and early antral follicles compared to early preantral follicles ( $p<0.01$ ) (Figure 6.1E). In the thecal layer, there was no change in the intensity of Tie-2 expression between early preantral, preantral and antral follicles (Figure 6.1F).

RT-PCR was performed to detect expression of Tie-2 mRNA in granulosa cell samples ( $n=4$  animals). Results are expressed as relative amount compared to expression of 18S and show that Tie-2 mRNA was expressed in granulosa cells, confirming that Tie-2 is expressed in granulosa cells (Figure 6.2).



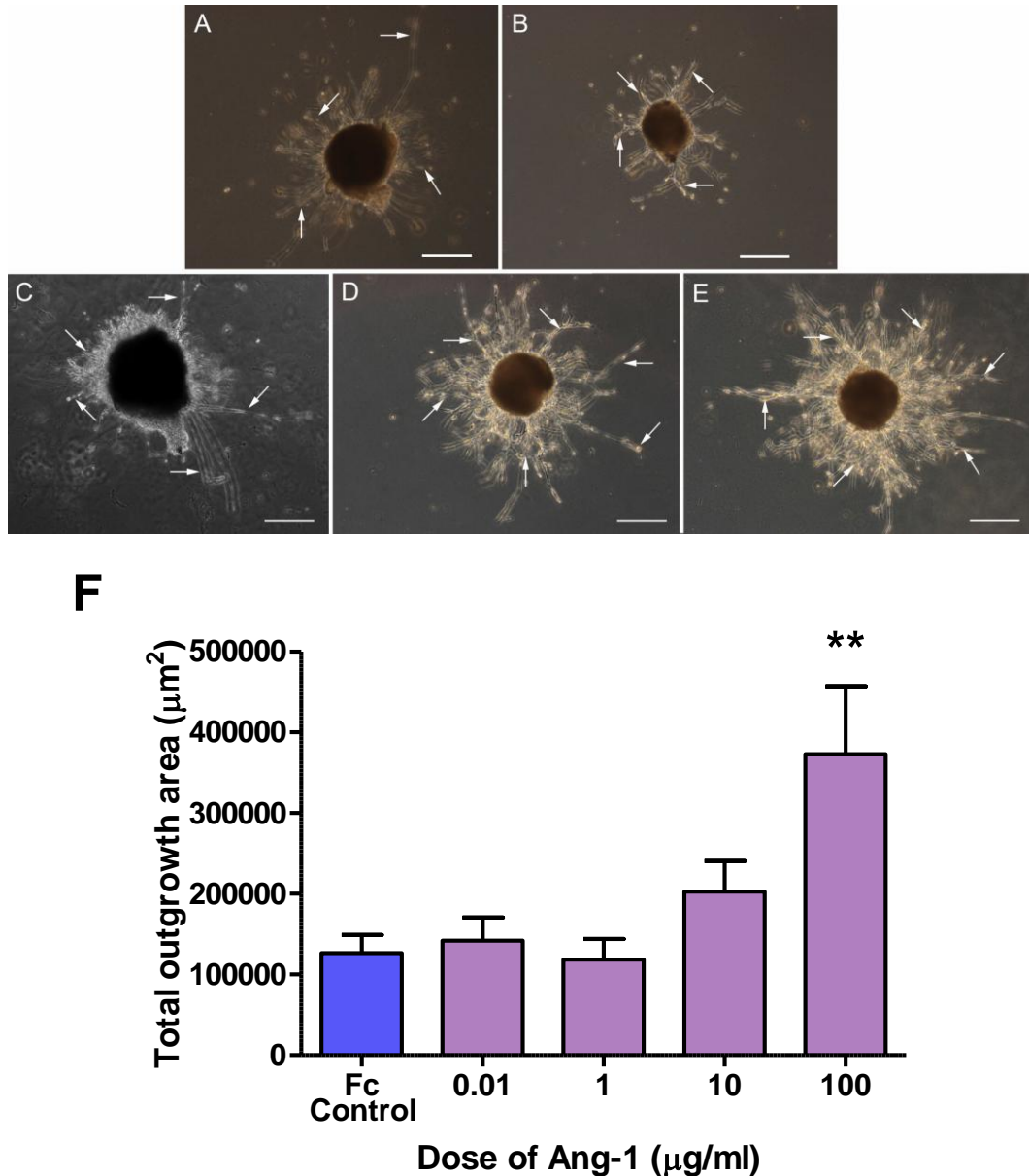


**Figure 6.2 Expression of Tie-2 mRNA in granulosa cells**

To confirm the expression of Tie-2 in granulosa cells, granulosa cells were cultured and then RNA was isolated for PCR. Expression of Tie-2 mRNA, in granulosa cell samples ( $n=4$  animals), was quantified by qRT-PCR and 18s mRNA as an internal control. Tie-2 mRNA was found to be expressed in granulosa cells. Experiments were repeated in triplicate.

### 6.3.2 Effect of Ang-1 on angiogenesis

To investigate the effect of Ang-1 on follicular angiogenesis, follicles were cultured in serum-free EBM-2 medium treated with 0.01, 1, 10 and 100  $\mu\text{g/ml}$  Ang-1 for six days. Treatment with Ang-1 resulted in a stimulation of follicular angiogenesis, in a dose dependent manner, (Figure 6.3) ( $P<0.01$ ), showing that Ang-1 is able to induce angiogenesis in whole ovarian follicles *in vitro*.

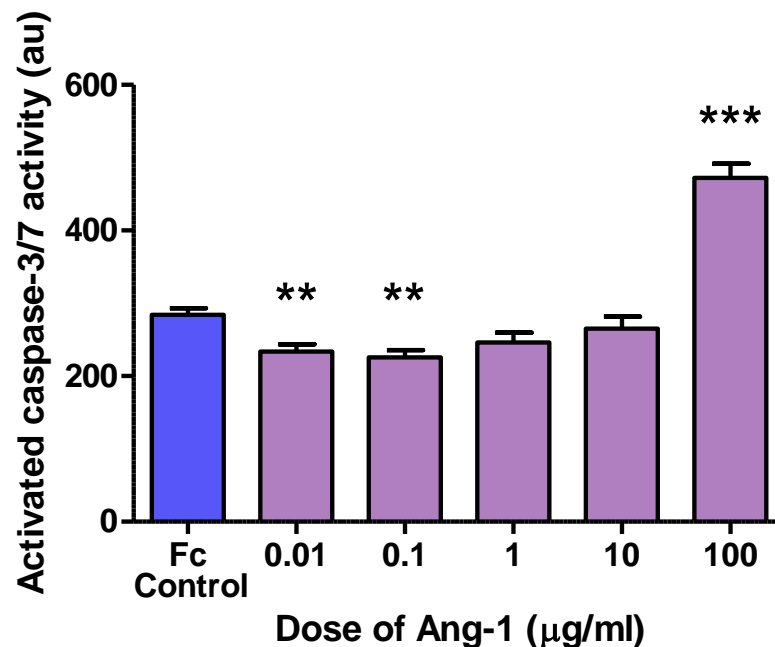


### Figure 6.3 Effect of Ang-1 on angiogenesis

To determine the effect of Ang-1 on angiogenesis isolated preantral and early antral follicles were cultured with varying doses of Ang-1 ( $n=6$  per treatment). At the end of the culture period the area of the angiogenic outgrowths was measured using Image Pro-Plus software. Representative images show endothelial outgrowths from a control follicle (A) and follicles after treatment with Ang-1 at 0.01 µg/ml (B), 1 µg/ml (C), 10 µg/ml (D) or 100 µg/ml (E). The results were quantified and analysed statistically using a One-way ANOVA with a Bonferroni post-hoc test to compare the Ang-1 treated follicles to the Fc control follicles. Treatment with 100 µg/ml Ang-1 resulted in a significant increase (\*\* $P<0.01$ ) in outgrowth area (F), when compared to the Fc control. White arrows indicate the endothelial outgrowths. Scale bars represent 200 µm. Experiments were repeated in triplicate.

### 6.3.3 Effect of Ang-1 on follicle health

To investigate the effect of Ang-1 on follicular apoptosis, isolated granulosa cells were cultured with 0, 0.01, 0.1, 1, 10 and 100 µg/ml Ang-1 for 48h followed by quantification of apoptosis using a luminescent caspase-3/7 assay. Activated caspase-3/7 activity was significantly decreased ( $P<0.01$ ) in the presence of low doses of Ang-1 (0.01 and 0.1 µg/ml), compared to controls. In contrast, treatment with 100 µg/ml Ang-1 resulted in a significant increase ( $P<0.001$ ) in caspase-3/7 activity (Figure 6.4).



**Figure 6.4 Effect of Ang-1 on granulosa cell apoptosis**

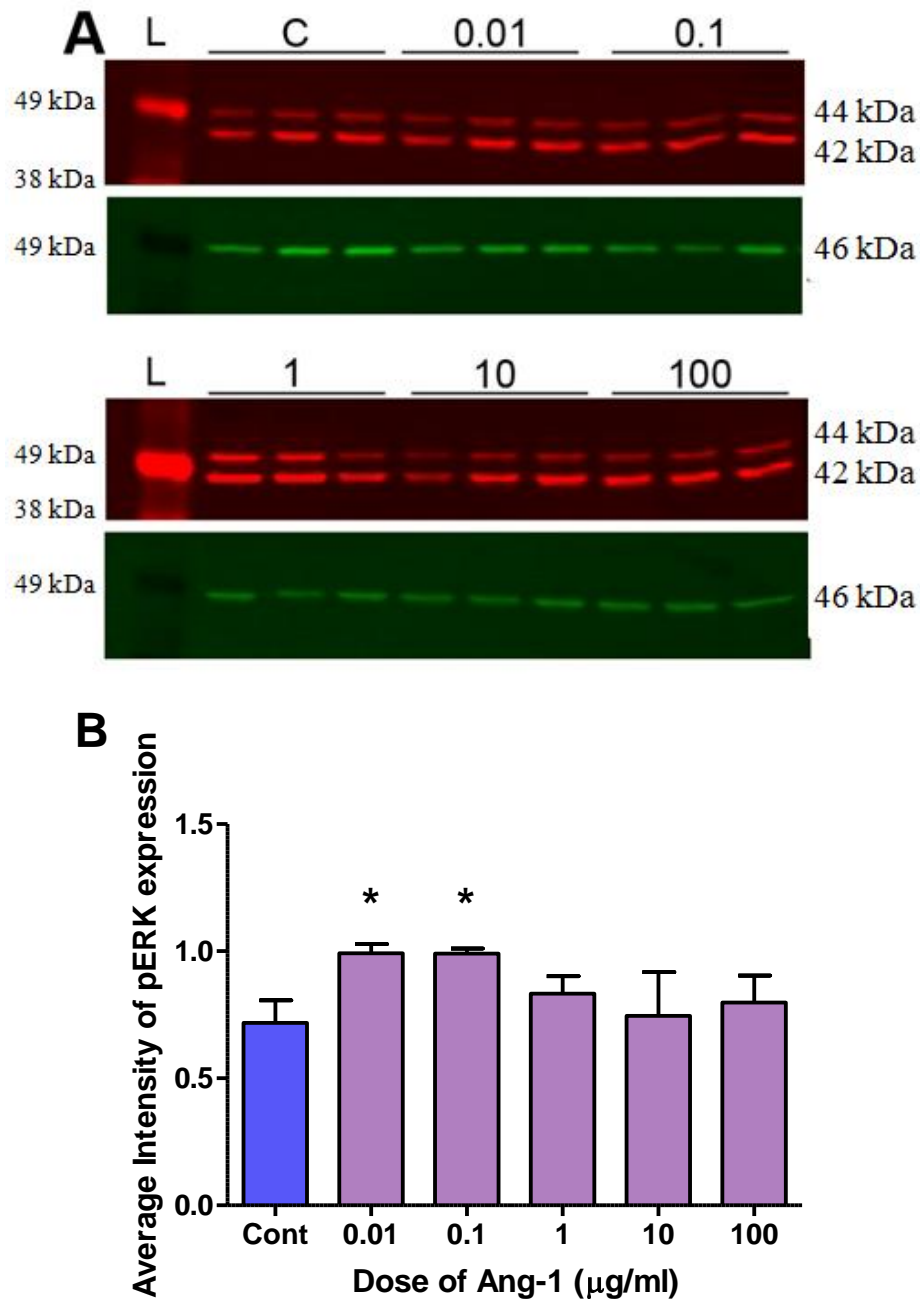
To determine the effect of Ang-1 on apoptosis granulosa cells were isolated from preantral and early antral follicles and they were cultured with varying doses of Ang-1 ( $n=4$  per treatment). At the end of the culture period these granulosa cells were analysed for expression of activated caspase-3/7 activity using a luminescent caspase activity assay, as an indicator of follicle health. A One-way ANOVA with a Bonferroni post-hoc test was performed to compare the Ang-1 treated granulosa cells to the Fc control granulosa cells. Treatment with 0.01 and 0.1 µg/ml Ang-1 resulted in a significant decrease ( $**P<0.01$ ) in activated caspase-3 expression ( $F$ ), when compared to the Fc control. In contrast, treatment with 100 µg/ml Ang-1 resulted in a significant increase ( $***P<0.001$ ) in activated caspase-3 expression ( $F$ ), when compared to the Fc control. Luminescence is given in arbitrary units (au). Experiments were repeated in triplicate.

### **6.3.4 Mechanisms of the pro-survival and apoptotic effects of Ang-1**

#### ***6.3.4.1 Quantification of pERK and p38 expression by western blotting***

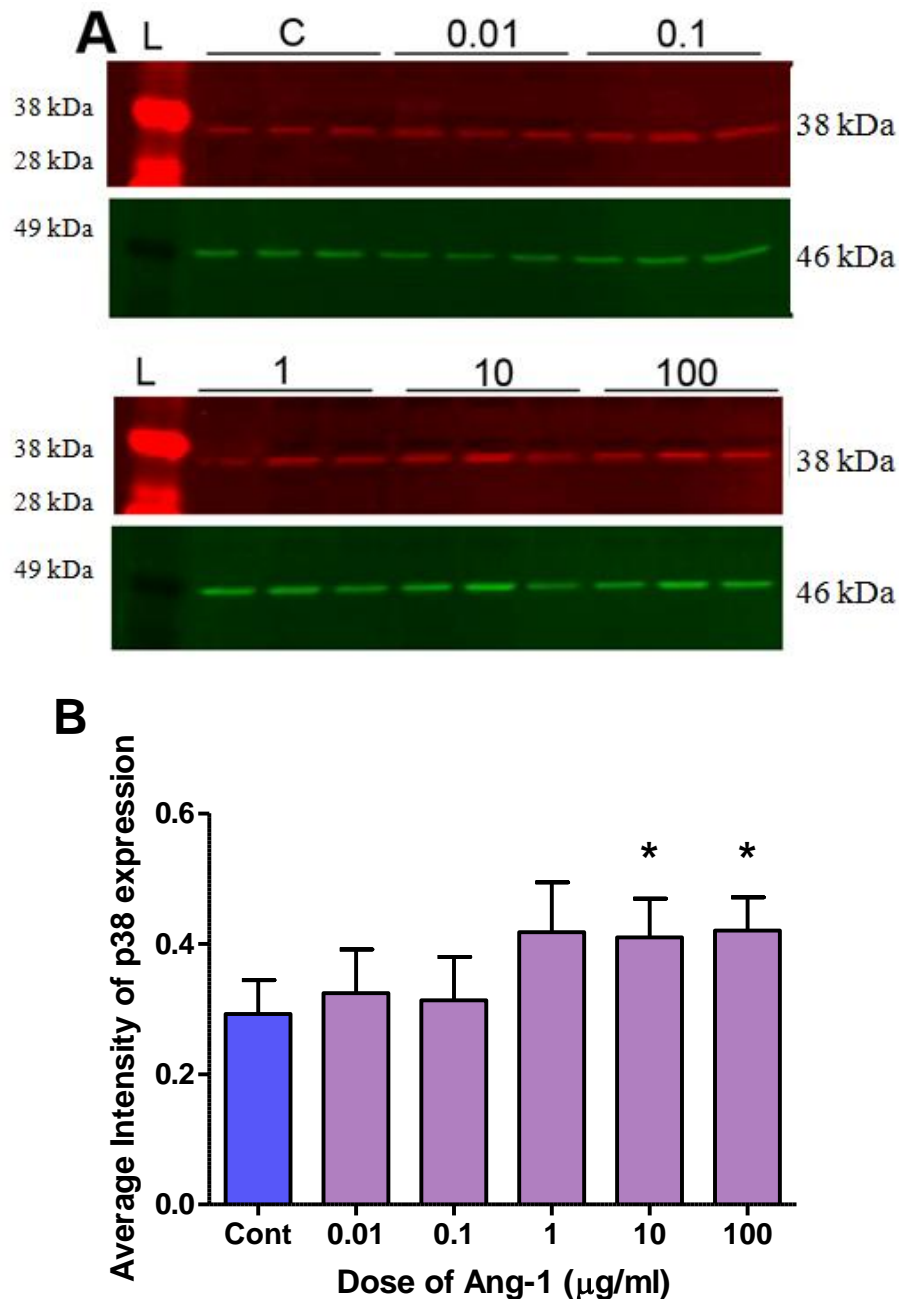
As we have shown that low doses of Ang-1 decrease expression of activated caspase-3 and that high doses can increase expression of activated caspase-3 the role of Ang-1 in promoting expression of signalling molecules involved in cell survival or apoptosis was investigated. To investigate this isolated granulosa cells were cultured with 0, 0.01, 0.1, 1, 10 and 100µg/ml of Ang-1 for 48h followed by quantification of the expression of pERK (Figure 6.5A) and p38 (Figure 6.6A) by western blotting. Consistent with the finding that low doses of Ang-1 reduced activated caspase-3 activity, the lowest doses of Ang-1 (0.01 and 0.1µg/ml) increased the expression of phospho-ERK1/2 (Figure 6.5B). In contrast, higher doses (10 and 100µg/ml) of Ang-1 increased phospho-p38 MAPK expression (Figure 6.6B).





**Figure 6.5 Levels of pERK expression after treatment with Ang-1**

Isolated granulosa cells were cultured with varying doses of Ang-1 ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed. To try to determine a potential mechanism of apoptosis induction the level of pERK expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with Ang-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA with a Bonferroni post-hoc test to compare the Ang-1 treated granulosa cells to the Fc control granulosa cells. Treatment with 0.01 and 0.1 µg/ml Ang-1 resulted in a significant increase ( $*P<0.05$ ) in the expression of pERK (F), when compared to the Fc control. Experiments were repeated in triplicate.

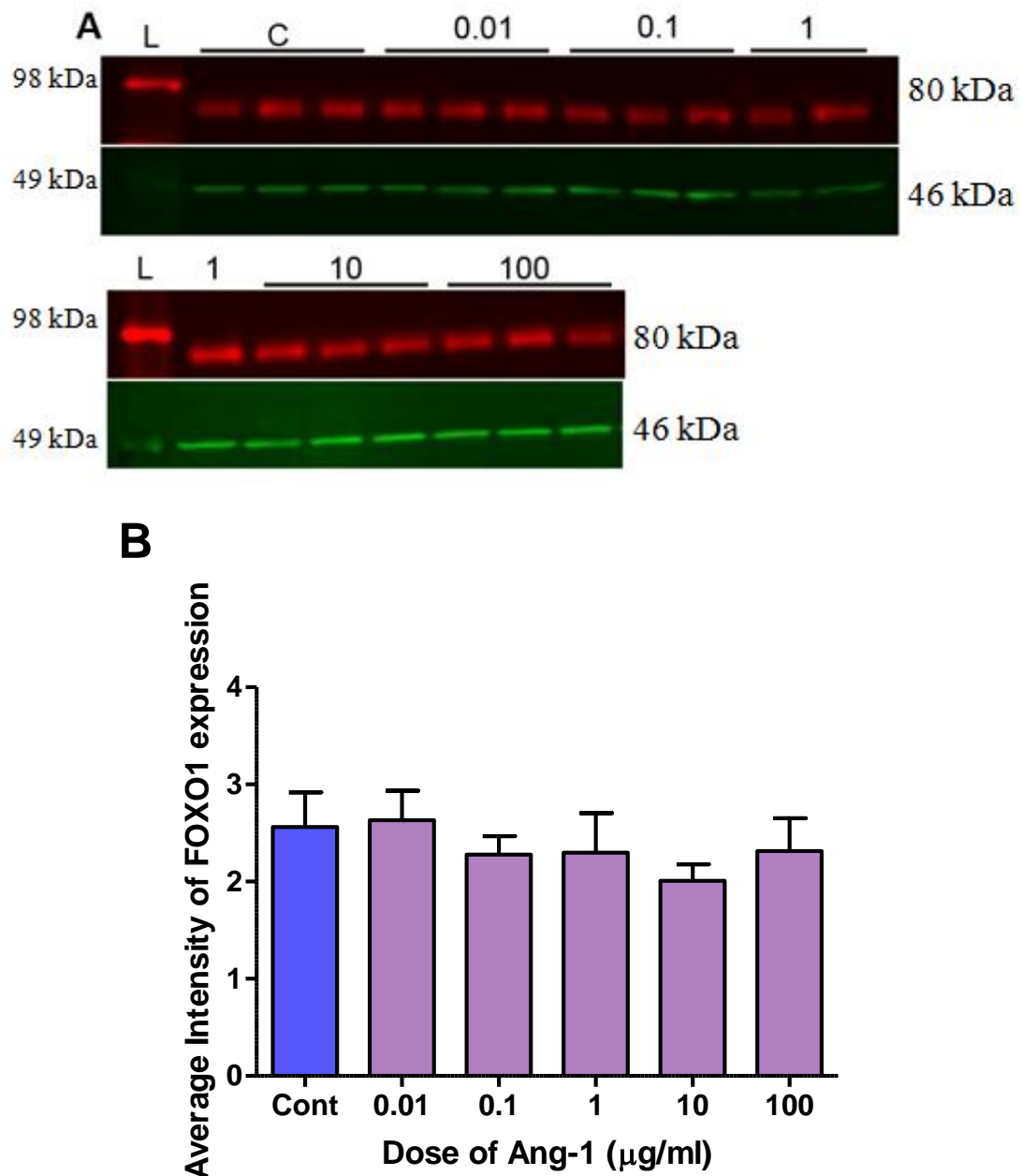


**Figure 6.6 Levels of p38 expression after treatment with Ang-1**

Isolated granulosa cells were cultured with varying doses of Ang-1 ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed. To try to determine a potential mechanism of apoptosis induction the level of p38 expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with Ang-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA with a Bonferroni post-hoc test to compare Ang-1 treated granulosa cells to the Fc control granulosa cells. Treatment with 10 and 100  $\mu\text{g/ml}$  Ang-1 resulted in a significant increase ( $*P<0.05$ ) in the expression of p38 (F), when compared to the Fc control. Experiments were repeated in triplicate.

#### **6.3.4.2 Quantification of *FOXO1* expression by western blotting**

FOXO1 is a transcription factor that blocks cell division and promotes apoptosis and is inactivated by Akt signalling. As Ang-1 has been shown to activate Akt we suggested that treatment with Ang-1 could lead to the inhibition of FOXO1 and therefore promote follicle health. To investigate this isolated granulosa cells were treated with 0, 0.01, 0.1, 1, 10 or 100µg/ml Ang-1 and then the level of FOXO1 expression was measured by western blotting (Figure 6.7A). The results showed that there was no difference in the expression of FOXO1 after treatment with Ang-1 (Figure 6.7B).



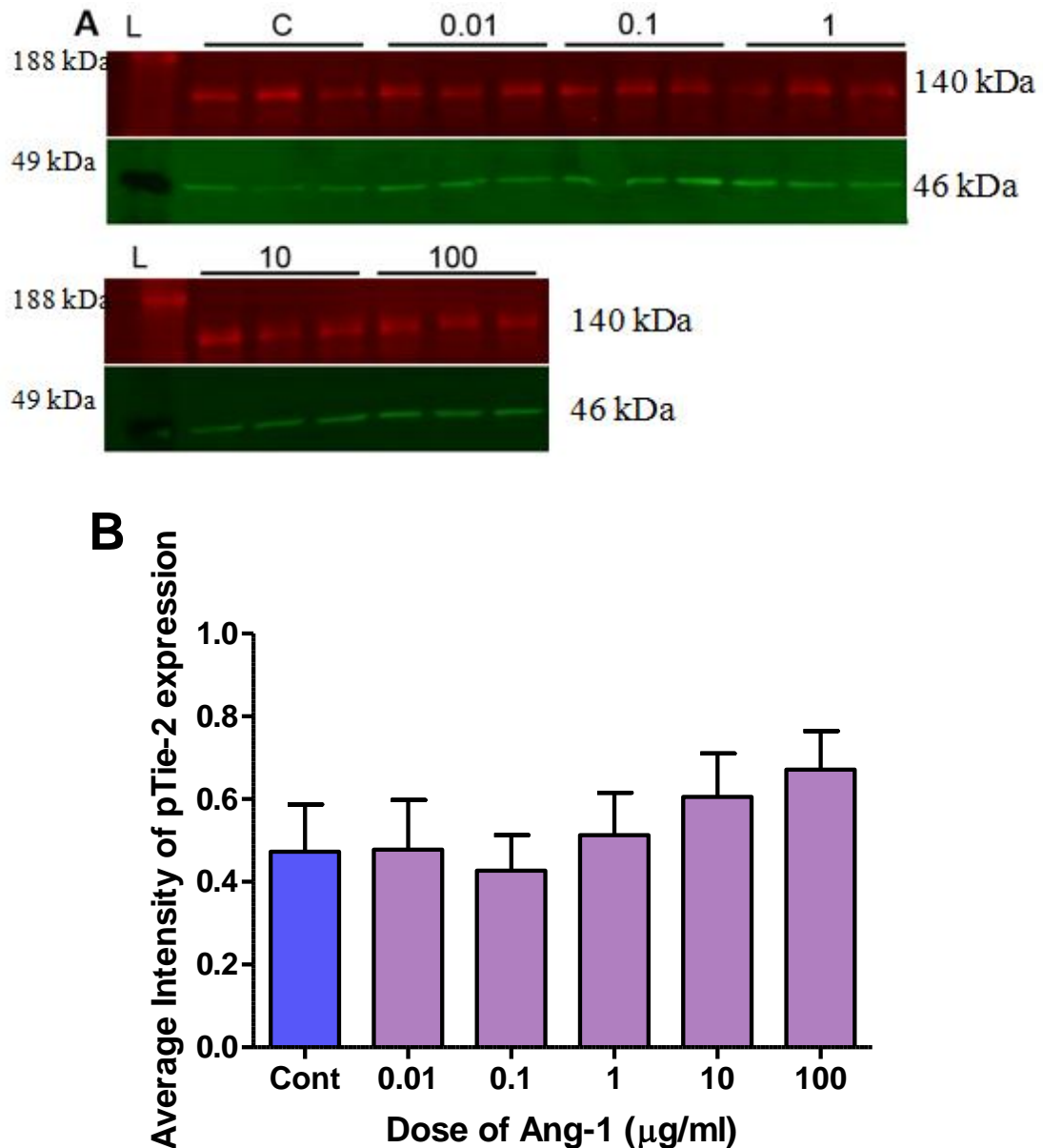
**Figure 6.7 Levels of FOXO1 expression after treatment with Ang-1**

Isolated granulosa cells were cultured with varying doses of Ang-1 ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed. To try to determine a potential mechanism of apoptosis induction the level of FOXO1 expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with Ang-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA to compare Ang-1 treated granulosa cells to the Fc control granulosa cells. The results show that there is no change in the expression of FOXO1 after treatment with Ang-1. Experiments were repeated in triplicate.

### **6.3.5 Mechanisms of the pro-angiogenic effect of Ang-1**

#### ***6.3.5.1 Quantification of pTie-2 expression by western blotting***

As Ang-1 is thought to promote blood vessel stabilisation through signalling via the Tie-2 receptor, the expression of phosphorylated Tie-2 (pTie-2) was quantified by western blotting (Figure 6.8A). These results showed that there was a trend of increasing pTie-2 expression in granulosa cells treated with increasing doses of Ang-1; however this was not significant (Figure 6.8B). This suggests that with the higher doses of Ang-1 there may be increased signalling via the Tie-2 receptor, giving support to the suggestion that Ang-1 promotes blood vessel development through activation of Tie-2.

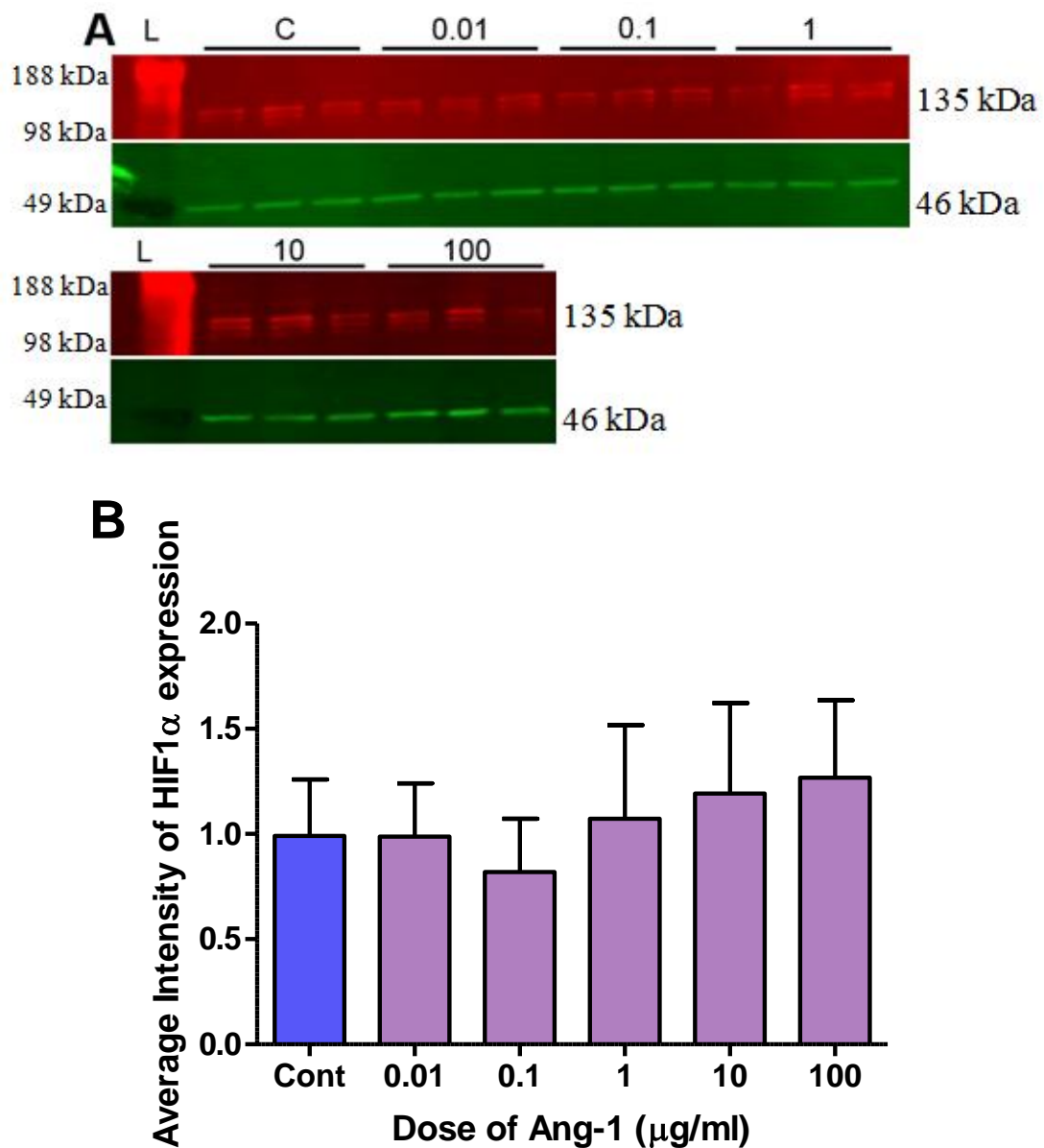


**Figure 6.8 Levels of pTie-2 expression after treatment with Ang-1**

Isolated granulosa cells were cultured with varying doses of Ang-1 ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed. To try to determine a potential mechanism of Ang-1 action on angiogenesis the level of pTie-2 expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with Ang-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA to compare Ang-1 treated granulosa cells to the Fc control granulosa cells. A trend in increasing expression of pTie-2 can be seen in granulosa cells treated with Ang-1. Experiments were repeated in triplicate.

**6.3.5.2 Quantification of HIF1 $\alpha$  expression by western blotting**

Hypoxic conditions are a key stimulator of angiogenesis and lead to increased expression of HIF1 $\alpha$ . The higher doses of Ang-1 have been shown to stimulate angiogenesis *in vitro* so the effect of Ang-1 treatment on the expression of HIF1 $\alpha$  was investigated to elucidate the mechanisms of the stimulation of angiogenesis by Ang-1. The results showed that there was no difference in HIF1 $\alpha$  expression after treatment with Ang-1 (Figure 6.9).



**Figure 6.9 Levels of HIF1 $\alpha$  expression after treatment with Ang-1**

Isolated granulosa cells were cultured with varying doses of Ang-1 ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed. To try to determine a potential mechanism of anti-angiogenesis the level of hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with Ang-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA to compare the Ang-1 treated granulosa cells to the Fc control granulosa cells. The results show that there is no change in the expression of HIF1 $\alpha$  after treatment with Ang-1. Experiments were repeated in triplicate.



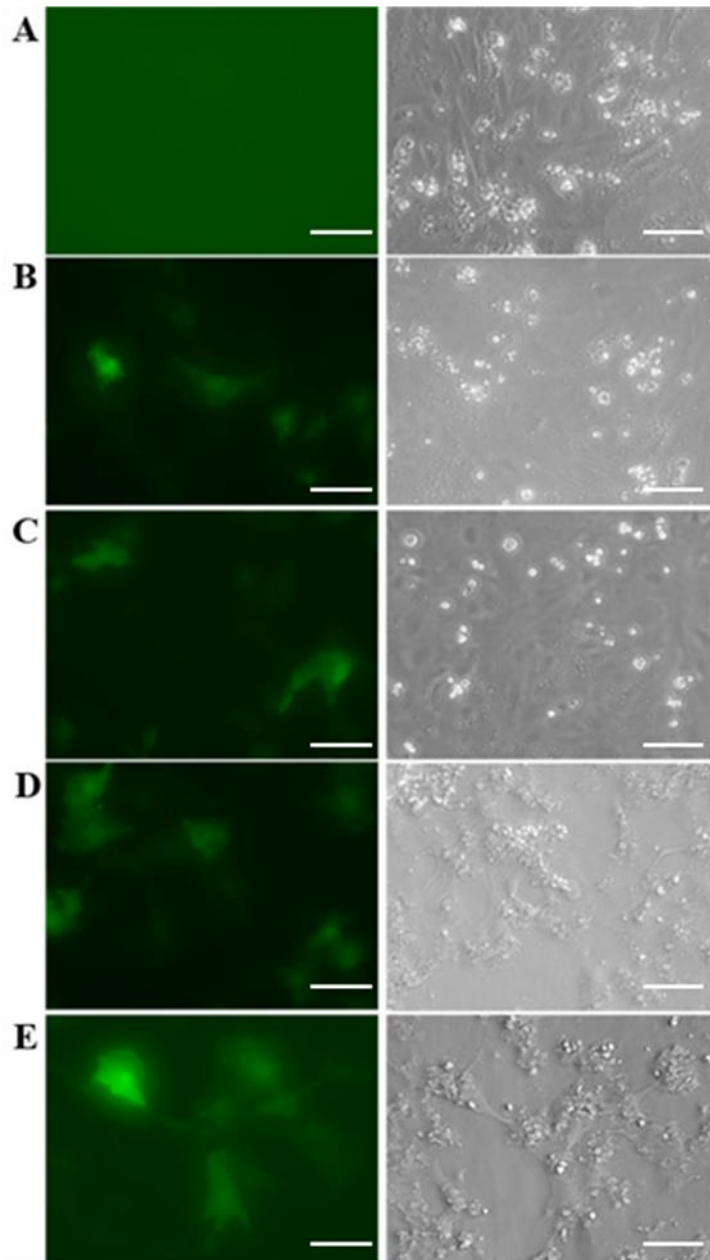
### **6.3.6 Investigation of the effect of RNA knockdown on genes of interest in rat granulosa cells**

To further confirm the role of Ang-1 in angiogenesis and cell survival and the pathways through which they occur, the effect of knocking down Tie-1, Tie-2 or Ang-2 was investigated using miRNA. Ang-1 acts mainly through binding to the receptor Tie-2 so it was hypothesized that knocking down the expression of the Tie-2 receptor would inhibit the actions of Ang-1. The receptor Tie-1 plays a role in mediating the actions of Ang-1 and Ang-2 but the importance of its role in this pathway is still unclear so Tie-1 was knocked down to determine whether the effects of Ang-1 were altered by the absence of Tie-1. Ang-2 acts in concert with Ang-1 in a competitive manner via the receptor Tie-2 so it was hypothesised that knocking down Ang-2 could result in increased activity of Ang-1 due to the lack of competition for the receptor.

#### ***6.3.6.1 Trial with GFP-expressing lentivirus***

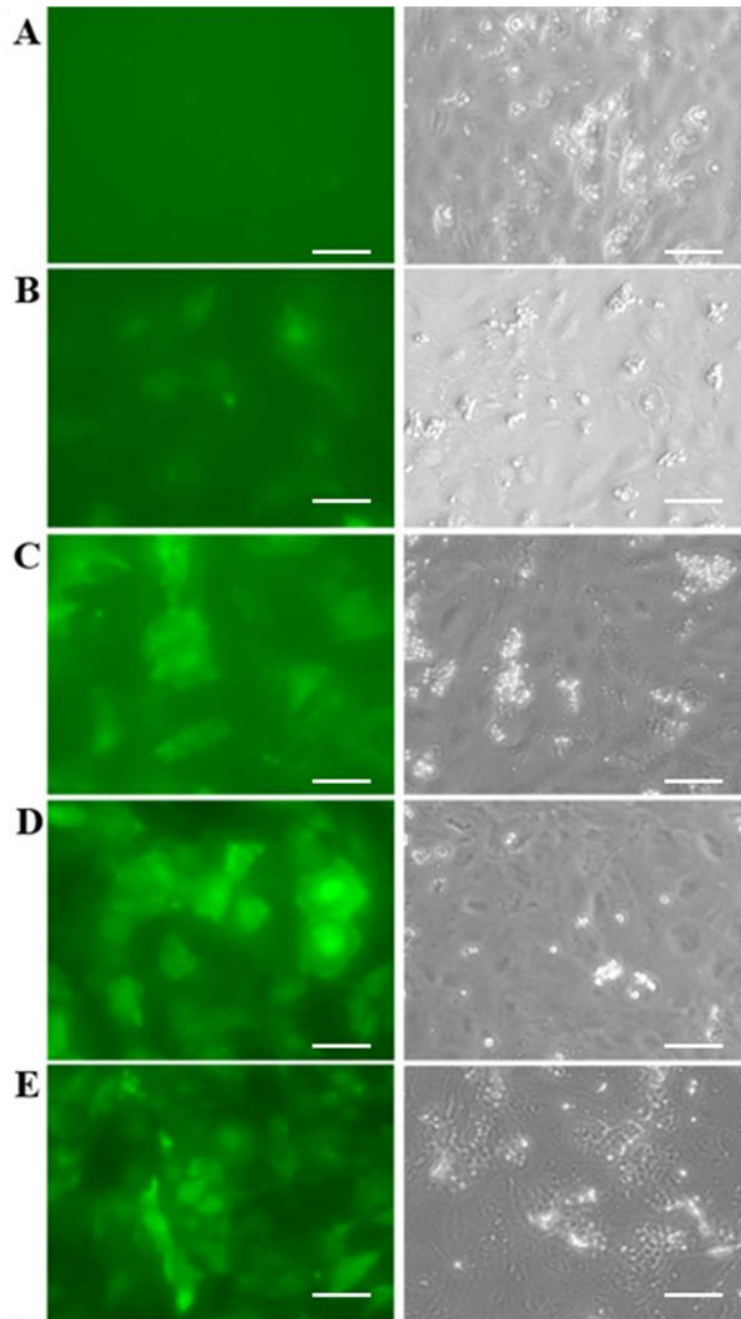
First, isolated granulosa cells were cultured with GFP-expressing lentivirus to confirm that the lentivirus would be incorporated into this cell type and also to establish the conditions required for efficient transfection. The granulosa cells were cultured for either 24 or 48h before the addition of the lentivirus and the lentivirus was removed from the cells after 8h. The lentivirus was added to the cells at varying concentrations (1 virus/cell, 5 viruses/cell, 10 viruses/cell and 50 viruses/cell) to determine the optimum virus to cell ratio. The level of GFP expression was monitored over 5 days to determine the optimal time for RNA and protein extraction. Granulosa cells cultured for 24h prior to the addition of the lentivirus did not efficiently incorporate the lentivirus (Figure 6.10) and few GFP-expressing granulosa

cells were observed. Granulosa cells cultured for 48h prior to the addition of the lentivirus efficiently incorporated the lentivirus with about 85-90% of granulosa cells expressing GFP after 72h (Figure 6.11). In addition, approximately 85-90% of granulosa cells were observed to be expressing GFP 96h after the removal of the lentivirus (Figure 6.12), suggesting that a longer culture post-lentivirus treatment is not necessary. Therefore the optimum conditions for the incorporation of lentivirus into granulosa cells were determined to be 48h culture prior to the addition of the lentivirus, 8h culture with the lentivirus at 10 viruses/cell, and then a further 72h culture prior to RNA or protein extraction.



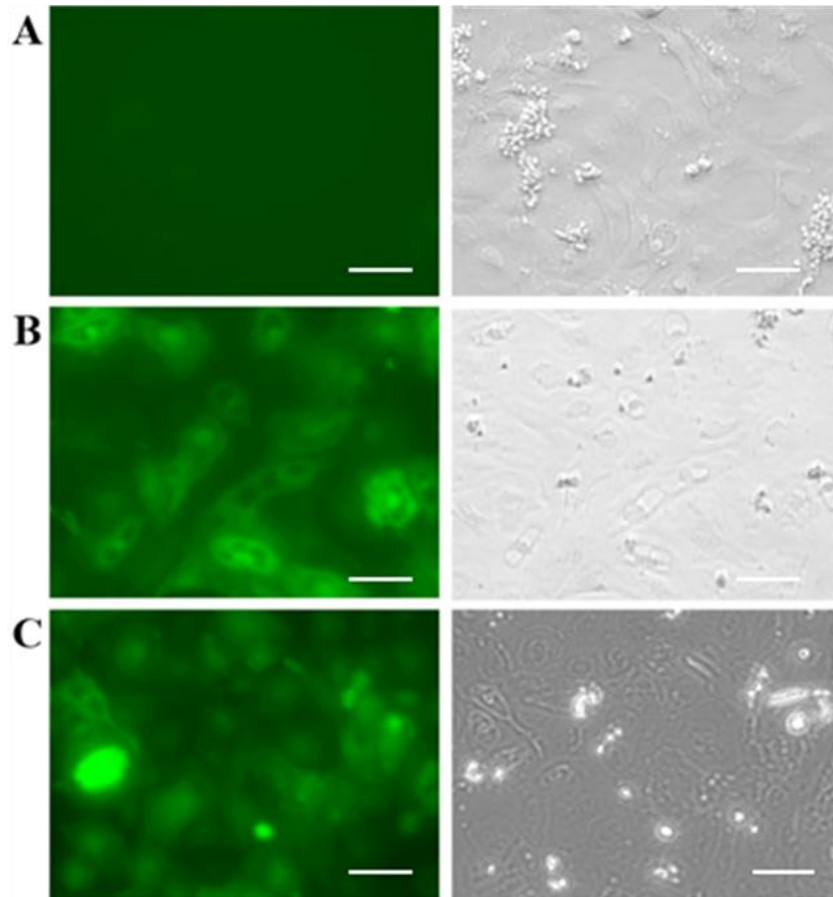
**Figure 6.10 Levels of GFP expression in granulosa cells cultured for 24h prior to lentiviral treatment**

*To investigate the effects of knocking down genes of interest on Ang-Tie signalling miRNA to these genes were transfected into GFP-expressing lentivirus. First it was determined whether the lentivirus would transfect into the isolated granulosa cells after being cultured for 24h prior to the addition of varying concentrations of GFP-expressing lentivirus and the level of GFP expression was detected after 72h. Representative fluorescent and brightfield images show granulosa cells cultured with a negative control lentivirus (A), with GFP-expressing lentivirus at 1 virus/cell (B), 5 viruses/cell (C), 10 viruses/cell (D) and 50 viruses/cell (E). Few GFP-expressing granulosa cells were observed even with 50 viruses/cell, suggesting that granulosa cells cultured for 24h prior to the addition of the lentivirus do not efficiently incorporate the lentivirus. Scale bars represent 50µm. Experiments were repeated in triplicate.*



**Figure 6.11 Levels of GFP expression in granulosa cells cultured for 48h prior to lentiviral treatment**

*To investigate the effects of knocking down genes of interest on Ang-Tie signalling miRNA to these genes were transfected into GFP-expressing lentivirus. First it was determined whether the lentivirus would transfect into the isolated granulosa cells after being cultured for 48h prior to the addition of varying concentrations of GFP-expressing lentivirus and the level of GFP expression was detected after 72h. Representative fluorescent and brightfield images show granulosa cells cultured with a negative control lentivirus (A), with GFP-expressing lentivirus at 1 virus/cell (B), 5 viruses/cell (C), 10 viruses/cell (D) and 50 viruses/cell (E). The results show that the lentivirus was incorporated into the granulosa cells and cells cultured with 10 viruses/cell or 50 viruses/cell had the highest percentage of GFP-expressing cells, with about 85-90% of granulosa cells expressing GFP after 72h. Scale bars represent 50µm. Experiments were repeated in triplicate.*

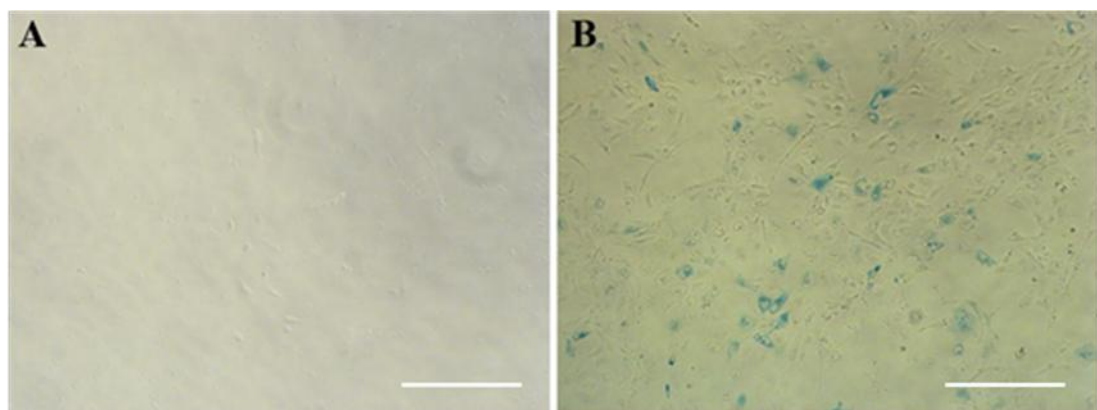


**Figure 6.12 Levels of GFP expression in granulosa cells cultured for 96h after lentiviral treatment**

*To determine the optimum time after lentiviral treatment for detection of GFP expression the level of GFP was detected at both 72hr (Figure 6.11) and 96hr. Isolated granulosa cells were cultured for 48h prior to the addition of varying concentrations of GFP-expressing lentivirus and the level of GFP expression was detected after 96h. Representative fluorescent and brightfield images show granulosa cells cultured with a negative control lentivirus (A), with GFP-expressing lentivirus at 10 viruses/cell (B) and 50 viruses/cell (C). The results show that the lentivirus was incorporated into the granulosa cells, with about 85-90% of granulosa cells expressing GFP after 96h. Scale bars represent 50µm. Experiments were repeated in triplicate.*

**6.3.6.2 Efficiency of transfection**

To assess the efficiency of transfection isolated granulosa cells were cultured with a LacZ-expressing lentivirus and then stained with X-gal. The results show that the lentivirus was incorporated into the granulosa cells, with about 35-40% of granulosa cells stained with X-gal (Figure 6.13). Due to the low titre of the lentivirus it was only possible to carry out this investigation with 1 virus/cell. It is expected that at a higher virus to cell ratio the efficiency of transfection would be greater and therefore should be sufficient to allow the knockdown of genes of interest to occur.



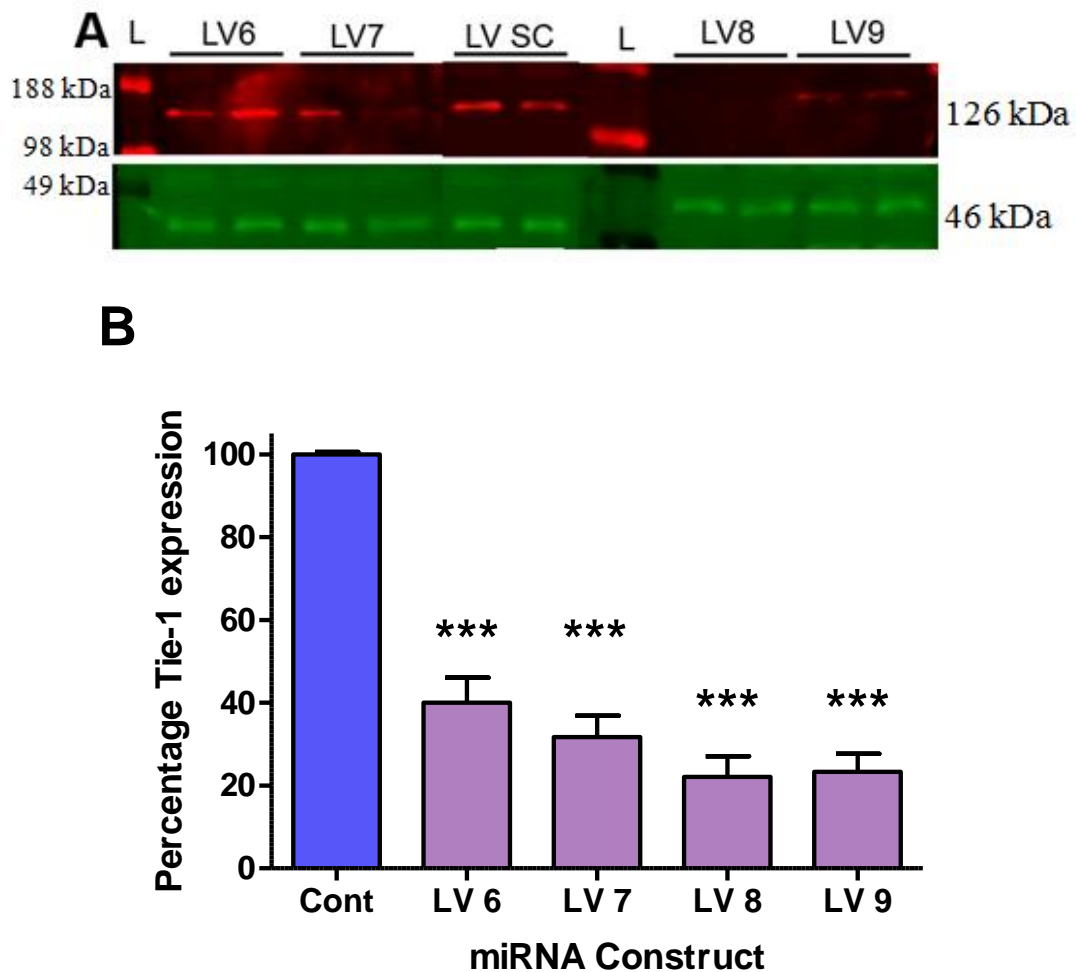
**Figure 6.13 Detection of X-gal staining in granulosa cells**

*To assess the efficiency of transfection, isolated granulosa cells were cultured with a LacZ-expressing lentivirus and then stained with X-gal. Isolated granulosa cells were cultured for 48h prior to the addition of LacZ-expressing lentivirus. After 72h X-gal was added to the cells to detect the expression of LacZ (blue staining). Representative images show granulosa cells cultured with a negative control lentivirus (A) or with LacZ-expressing lentivirus at 1 virus/cell (B). The results show that the lentivirus was incorporated into the granulosa cells, with about 35% of granulosa cells staining with X-gal. Scale bars represent 50µm. Experiments were repeated in triplicate.*

### **6.3.7 Effect of Tie-1 knockdown on VEGF signalling**

#### ***6.3.7.1 Trial of miRNA constructs designed to knockdown Tie-1***

It has been reported that VEGF is able to activate the Tie-2 receptor in HUVECS independently of Ang-1 binding via cleavage of Tie-1 (Singh et al., 2009). To investigate this, miRNA either specifically targeted to Tie-1 or with a scrambled sequence as a control, was transfected into granulosa cells using lentiviral delivery. As the miRNA constructs were cloned into a GFP-expressing lentivirus the level of incorporation into the granulosa cells could be detected by the level of GFP expression. All of the miRNA constructs that were designed to knockdown Tie-1 were highly incorporated into the granulosa cells, with approximately 85% of granulosa cells expressing GFP. Once this had been determined, the level of Tie-1 expression was quantified by western blotting (Figure 6.14A). The results show that there was a significant decrease in the expression of Tie-1 ( $P < 0.001$ ) after treatment with the miRNA constructs (Figure 6.14B), showing that the miRNA constructs were able to knockdown expression of Tie-1 in these cells. Constructs LV8 and LV9 gave the greatest reduction in expression of Tie-1 – approximately an 85% knockdown of expression, so they were used in further experiments.



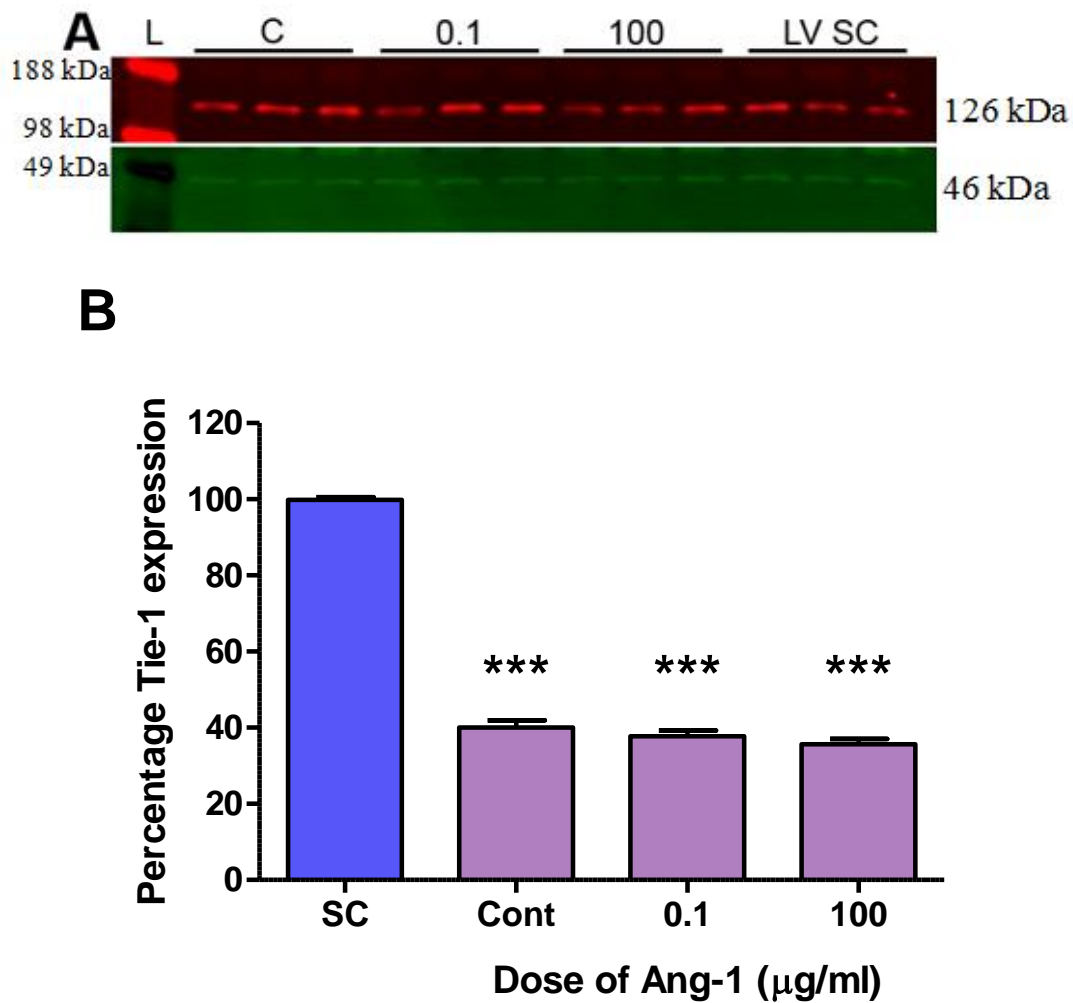
**Figure 6.14 Levels of Tie-1 expression after treatment with miRNA**

To determine whether the miRNA constructs were able to knock down the expression of Tie-1 isolated granulosa cells were cultured with various miRNA constructs ( $n=3$  per treatment) designed to knock-down Tie-1. At the end of the culture protein was extracted from the granulosa cells and the level of Tie-1 expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with Ang-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA with a Bonferroni post-hoc test to compare the effect of the different constructs to the control granulosa cells. The results show that there is a significant decrease ( $***P<0.001$ ) in the expression of Tie-1 after treatment with the miRNA constructs when compared to the control. Experiments were repeated in triplicate.



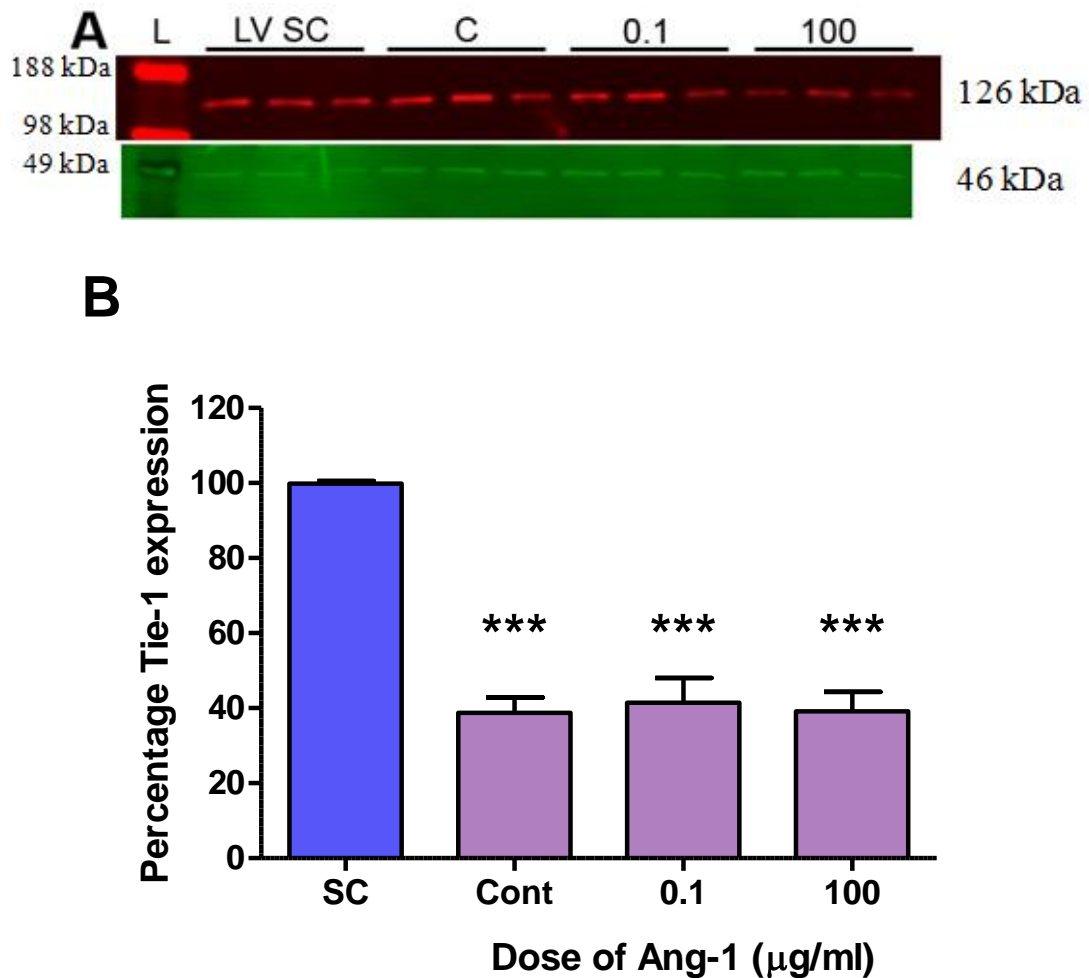
**6.3.7.2 Effect of Tie-1 knockdown on expression of VEGF**

Constructs LV8 and LV9 gave the greatest reduction in expression of Tie-1 so they were used to investigate the effect of Tie-1 knockdown on VEGF signalling. Isolated granulosa cells were treated with constructs LV8 and LV9 and with varying doses of Ang-1. First, westerns for Tie-1 were performed to confirm that miRNA LV8 and LV9 knocked down expression of Tie-1. The results showed that there is a significant decrease in Tie-1 expression after treatment with LV8 (Figure 6.15) and with LV9 (Figure 6.16). However, there was no difference in the level of knock down of Tie-1 expression after treatment with Ang-1.



**Figure 6.15 Levels of Tie-1 expression after treatment with Ang-1 and LV8**

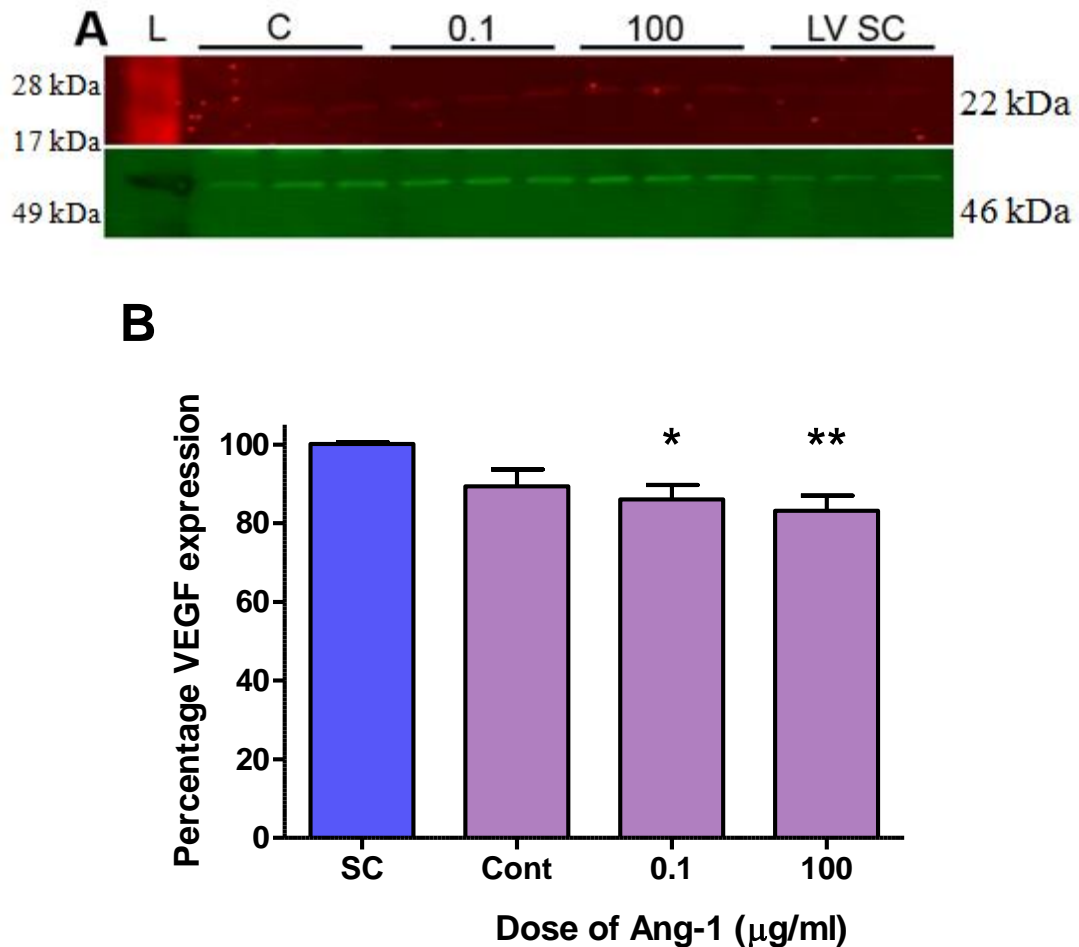
Once it had been determined that miRNA construct LV8 sufficiently knocked down Tie-1 expression the effect of knocking down Tie-1 expression on the effect of Ang-1 was investigated. Isolated granulosa cells were cultured with miRNA construct LV8 designed to knock-down Tie-1 along with 0, 0.1, or 100 μg/ml Ang-1 ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed and then the level of Tie-1 expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with Ang-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA with a Bonferroni post-hoc test to compare the Ang-1 treated granulosa cells to the scrambled control granulosa cells. The results show that there is a significant decrease ( $***P<0.001$ ) in the expression of Tie-1 after treatment with LV8 when compared to the scrambled control. There was no difference in the level of knock down of Tie-1 expression after treatment with Ang-1. Experiments were repeated in triplicate.



**Figure 6.16 Levels of Tie-1 expression after treatment with Ang-1 and LV9**

Once it had been determined that miRNA construct LV9 sufficiently knocked down Tie-1 expression the effect of knocking down Tie-1 expression on the effect of Ang-1 was investigated. Isolated granulosa cells were cultured with miRNA construct LV8 designed to knock-down Tie-1 along with 0, 0.1, or 100 µg/ml Ang-1 ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed and then the level of Tie-1 expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with Ang-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA with a Bonferroni post-hoc test to compare the Ang-1 treated granulosa cells to the scrambled control granulosa cells. The results show that there is a significant decrease ( $***P<0.001$ ) in the expression of Tie-1 after treatment with LV9, when compared to the scrambled control. There was no difference in the level of knock down of Tie-1 expression after treatment with Ang-1. Experiments were repeated in triplicate.

Subsequently westerns for VEGF were performed to investigate whether knockdown of Tie-1 expression had an effect on expression of VEGF. The results showed that there is a significant decrease ( $P<0.05$ ) in VEGF expression after treatment with the miRNA constructs LV8 and LV9 (Figure 6.17), but only after treatment with the 0.1 and 100 $\mu$ g/ml Ang-1.



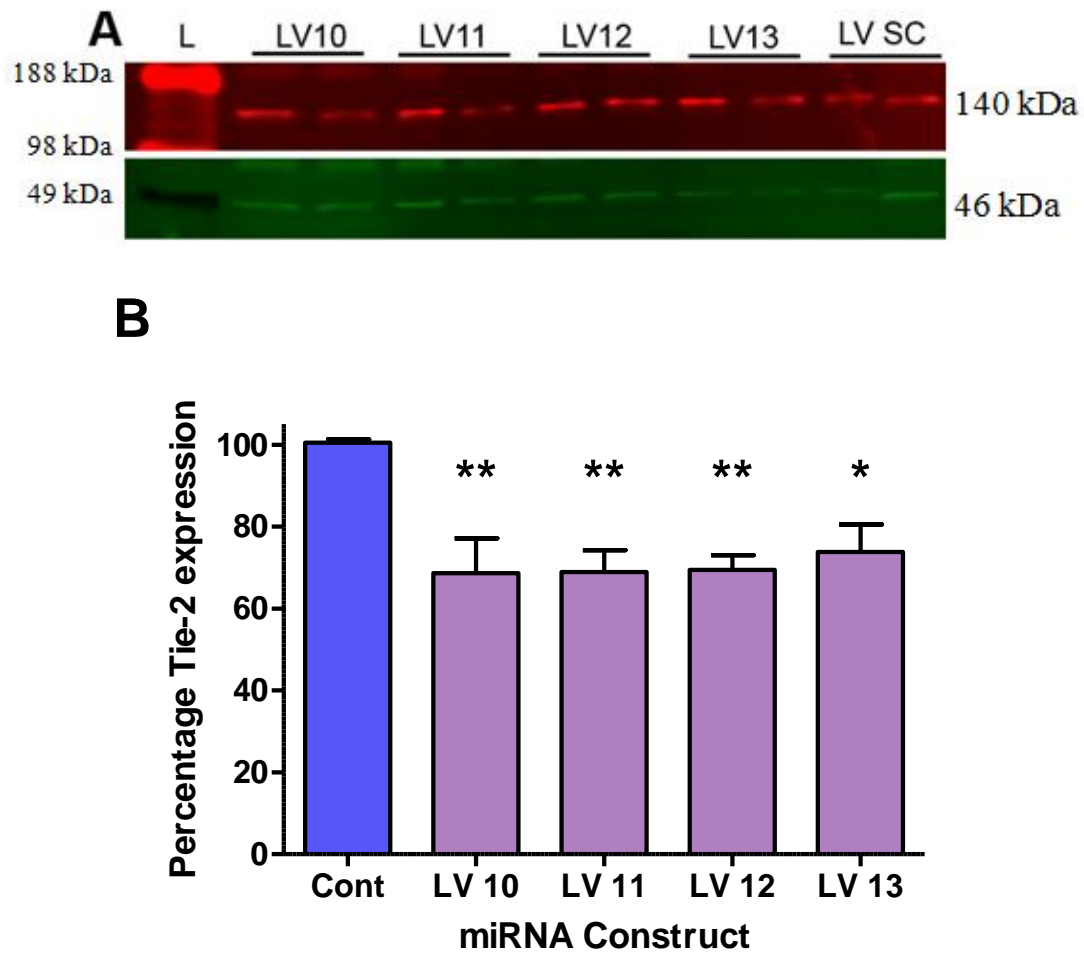
**Figure 6.17 Levels of VEGF expression after treatment with Ang-1**

Once it had been determined that the miRNA constructs sufficiently knocked down *Tie-1* expression the effect of knocking down *Tie-1* expression on VEGF expression was investigated. Isolated granulosa cells were cultured with miRNA construct LV8 or miRNA construct LV9 both designed to knock-down *Tie-1* along with 0, 0.1, or 100 µg/ml Ang-1 ( $n=3$  per treatment) and protein was extracted to allow western blotting to be performed and then the level of VEGF expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with Ang-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA with a Bonferroni post-hoc test to compare the effect of the different constructs to the scrambled control granulosa cells. Treatment with 0.1 and 100 ng/ml Ang-1 resulted in a statistically significant decrease in the expression of VEGF after knock down of *Tie-1*. \* indicates  $P<0.05$ , \*\* indicates  $P<0.01$ , when compared to the scrambled control. Experiments were repeated in triplicate.

### **6.3.8 Effect of Tie-2 knockdown**

#### ***6.3.8.1 Trial of miRNA constructs designed to knockdown Tie-2***

Ang-1 acts via the receptor Tie-2 to promote angiogenesis and cell survival so it is likely that with the removal of this receptor there would be a negative effect on cell survival and possibly a destabilisation of newly developed blood vessels. To investigate this miRNA either specifically targeted to Tie-2 or with a scrambled sequence as a control, was transfected into granulosa cells using lentiviral delivery and then the level of Tie-2 expression was quantified by western blotting (Figure 6.18A). These the results showed that there was a significant decrease in the expression of Tie-2 ( $P<0.05$ ) after treatment with the miRNA constructs (Figure 6.18B). However the level of knockdown of Tie-2 expression was only around 25% which was not sufficient to allow further investigations to be carried out.



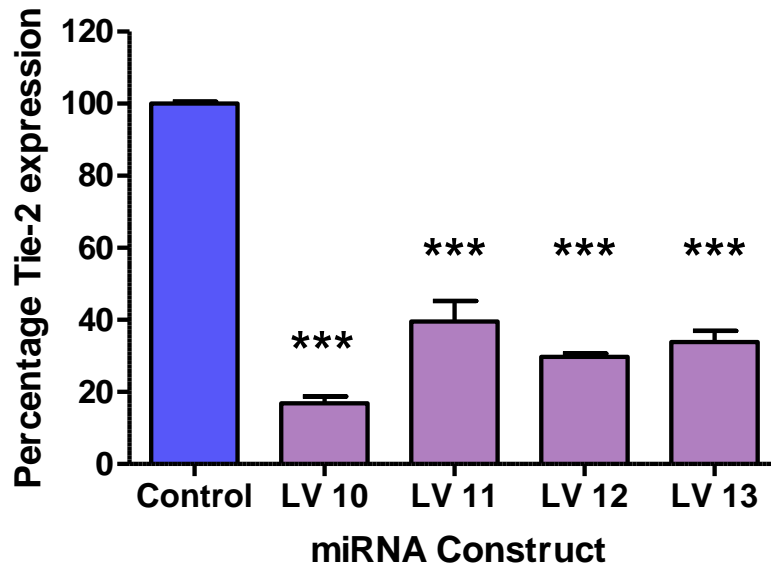
**Figure 6.18 Levels of Tie-2 expression after treatment with miRNA**

To determine whether the miRNA constructs were able to knock down the expression of Tie-2 isolated granulosa cells were cultured with various miRNA constructs ( $n=3$  per treatment). At the end of the culture protein was extracted from the granulosa cells and the level of Tie-2 expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with Ang-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA with a Bonferroni post-hoc test to compare the effect of the different constructs to the control granulosa cells. The results show that there is a statistically significant decrease in the expression of Tie-2 after treatment with the miRNA constructs. \* indicates  $P<0.05$ , \*\* indicates  $P<0.01$ , when compared to the control. Experiments were repeated in triplicate.

**6.3.8.2 Effect of Tie-2 knockdown on mRNA expression of Tie-2**

As the miRNA constructs were cloned into a GFP-expressing lentivirus the level of incorporation into the granulosa cells could be detected by the level of GFP expression. All of the miRNA constructs that were designed to knockdown Tie-2 were highly incorporated into the granulosa cells, with approximately 85% of granulosa cells expressing GFP. However the level of knockdown of Tie-2 expression was very low for this level of lentiviral incorporation into the cells. This suggests that the protein is very stable and changes in protein expression are not easily detectable. Therefore the expression of Tie-2 mRNA was determined by qRT-PCR to investigate whether knockdown of the gene had occurred (Figure 6.19). The results show that there is a significant decrease in the expression of Tie-2 mRNA ( $P < 0.001$ ) after treatment with all of the miRNA constructs and that constructs LV10 and LV12 gave the greatest reduction in expression of Tie-2 – approximately 75-85% knockdown of expression.





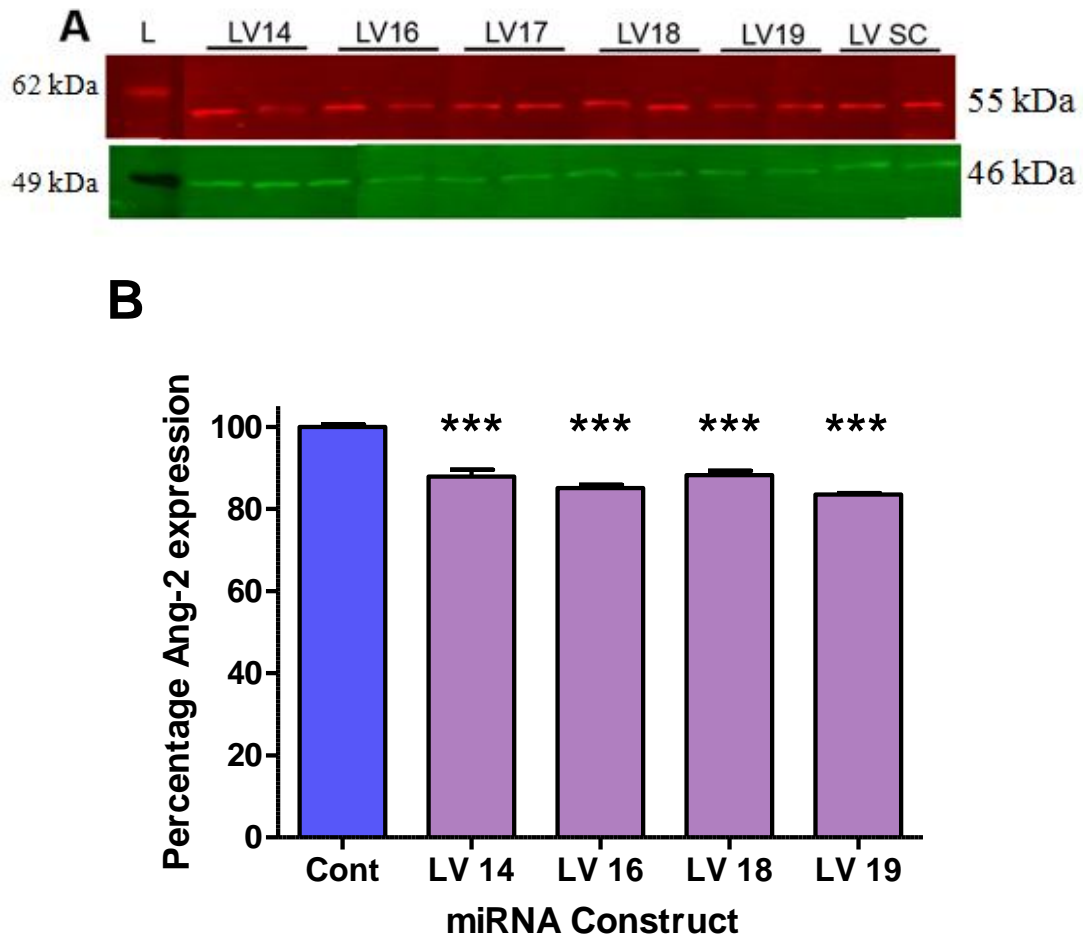
**Figure 6.19 Levels of Tie-2 mRNA expression after treatment with miRNA**

The level of knock-down of Tie-2 protein expression was not as expected when compared to the GFP expression in these cells so the level of Tie-2 mRNA was investigated to determine whether the miRNA constructs had knocked down the expression of Tie-2. Isolated granulosa cells were cultured with miRNA constructs designed to knock-down Tie-2 expression and then the level of Tie-2 expression was measured by qRT-PCR (A). These data were quantified (B) and analysed statistically by One-way ANOVA with a Bonferroni post-hoc test to compare the effect of the different constructs to the control granulosa cells. The results show that there is a statistically significant decrease ( $***P < 0.001$ ) in the expression of Tie-2 after treatment with the miRNA constructs, when compared to the control. Experiments were repeated in triplicate.

### **6.3.9 Effect of Ang-2 knockdown**

#### ***6.3.9.1 Trial of miRNA constructs designed to knockdown Ang-2***

Ang-2 binds to the receptor Tie-2 but unlike Ang-1 does not activate it, and excess Ang-2 inhibits the activity of Ang-1 (Maisonpierre et al., 1997). In addition, Ang-2 has been shown to be expressed in the rat ovary and is highly expressed in the granulosa cell layer of atretic follicles, suggesting that Ang-2 could both play a role in the regulation of angiogenesis and could have an extravascular role in the ovary. Therefore the effect of suppressing the expression of Ang-2 was investigated *in vitro*. Isolated granulosa cells were cultured with miRNA constructs designed to knockdown Ang-2 and then the level of Ang-2 expression was quantified by western blotting (Figure 6.20A). The results showed that there was a significant decrease in the expression of Ang-2 ( $P < 0.001$ ) after treatment with the miRNA constructs (Figure 6.20B). However the level of knockdown of Ang-2 expression was only around 15% which is not sufficient to allow further investigations to be carried out.

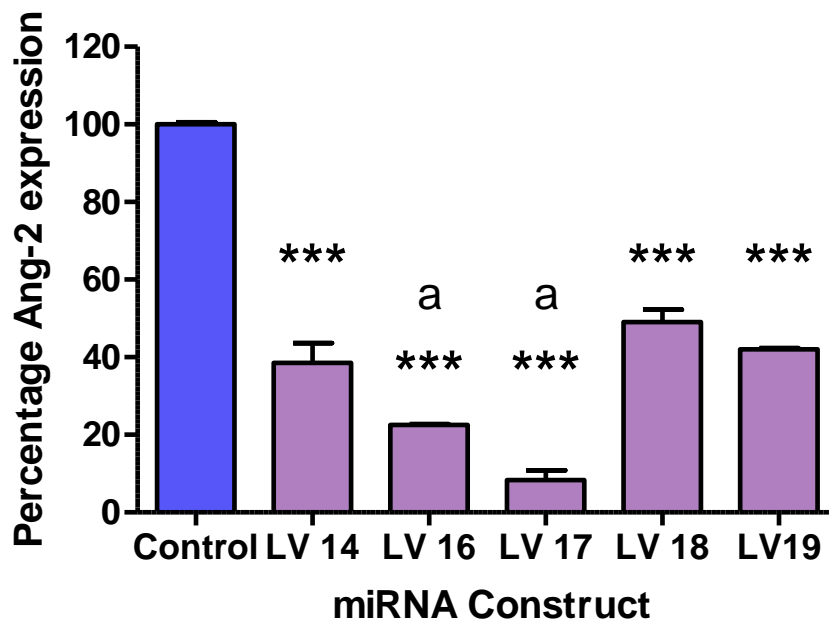


**Figure 6.20 Levels of Ang-2 expression after treatment with miRNA**

To determine whether the miRNA constructs were able to knock down the expression of Ang-2 isolated granulosa cells were cultured with various miRNA constructs ( $n=3$  per treatment). At the end of the culture protein was extracted from the granulosa cells and the level of Ang-2 expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with Ang-1. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA with a Bonferroni post-hoc test to compare the effect of the different constructs to the control granulosa cells. The results show that there is a slight, but significant, decrease ( $***P<0.001$ ) in the expression of Ang-2 after treatment with the miRNA constructs, when compared to the control. Experiments were repeated in triplicate.

**6.3.9.2 Effect of Ang-2 knockdown on mRNA expression of Ang-2**

As the miRNA constructs were cloned into a GFP-expressing lentivirus the level of incorporation into the granulosa cells could be detected by the level of GFP expression. All of the miRNA constructs that were designed to knockdown Ang-2 were highly incorporated into the granulosa cells, with approximately 85% of granulosa cells expressing GFP. However the level of knockdown of Ang-2 expression was very low for this level of lentiviral incorporation into the cells. This suggests that the protein is very stable and changes in protein expression are not easily detectable. Therefore the expression of Ang-2 mRNA was determined by qRT-PCR to investigate whether knockdown of the gene had occurred (Figure 6.21). The results show that there is a significant decrease in the expression of Ang-2 ( $P < 0.001$ ) after treatment with all of the miRNA constructs and that constructs LV16 and LV17 gave the greatest reduction in expression of Ang-2— approximately 80-90% knockdown of expression.



**Figure 6.21 Levels of Ang-2 mRNA expression after treatment with miRNA**

The level of knock-down of Ang-2 protein expression was not as expected when compared to the GFP expression in these cells so the level of Ang-2 mRNA was investigated to determine whether the miRNA constructs had knocked down the expression of Ang-2. Isolated granulosa cells were cultured with miRNA constructs designed to knock-down Ang-2 expression and then the level of Ang-2 expression was measured by qRT-PCR (A). These data were quantified (B) and analysed statistically by One-way ANOVA with a Bonferroni post-hoc test to compare the effect of the different constructs to the control granulosa cells. The results show that there is a significant decrease ( $***P < 0.001$ ) in the expression of Ang-2 after treatment with the miRNA constructs, when compared to the control. <sup>a</sup> indicates  $P < 0.05$ , in comparison with LV14, LV 18 and LV19. Experiments were repeated in triplicate.

## 6.4 Discussion

This chapter has shown that Ang-1 promotes follicular angiogenesis *in vitro* in a dose dependent manner. Furthermore, it has been shown for the first that Tie-2 is expressed in rat granulosa cells and that Ang-1 has a direct functional role in promoting granulosa cell survival *in vitro*, independent of its role in angiogenesis.

Ang-1 is known to be important for the development of new blood vessels and has been shown to influence the survival and apoptosis of endothelial cells. It has previously been shown that Ang-1 can stimulate endothelial cell proliferation *in vitro* (Koblizek et al., 1998). In this study it has been demonstrated that Ang-1 stimulates angiogenesis in the ovarian follicle, *in vitro*, however, the dose that stimulated angiogenesis is much higher than the expected physiological concentration of Ang-1 suggesting that this may be a pharmacological effect. Treatment of intact ovarian follicles with Ang-1 resulted in the development of angiogenic outgrowths and the use of the *in vitro* angiogenesis assay described in Chapter 3 allowed follicle health and angiogenesis to be quantified. Different stages of follicles were cultured and follicles at the early antral stage of development had a tendency to undergo increased angiogenesis when compared with smaller follicles, regardless of treatment. However, the level of angiogenesis undergone by all follicles was increased after treatment with Ang-1, in a dose dependent manner. Ang-1 is known to act through the receptor Tie-2 but the specific mechanism by which it promotes angiogenesis required further elucidation. This was investigated and there was a trend of increasing expression of pTie-2 as the dose of Ang-1 increased, suggesting that the

higher the level of Ang-1 stimulation, the higher the activation, and therefore phosphorylation, of the receptor Tie-2. This agrees with previous findings (Koblizek et al., 1998) and highlights the importance of signalling via Tie-2 in the induction of angiogenesis.

Ang-1 has been shown to be expressed in the rat ovary and has been localised to the thecal endothelium of rat ovarian follicles (Abramovich et al., 2009) with expression increasing as follicular development progresses. Ang-1 acts through the receptor Tie-2 and previous studies have localised Tie-2 expression to the thecal layer of ovarian follicles (Abramovich et al., 2009). This contrasts with the findings of this chapter as the expression of Tie-2 was detected and quantified by immunohistochemistry and was found to be expressed in both the thecal and granulosa cell layers of rat ovarian follicles. The process of fixing and embedding tissues can mask antigenic epitopes so antigen retrieval by heat treatment (Shi et al., 1991) was performed to ensure that this did not occur. Abramovich *et al* (Abramovich et al., 2009) showed that Tie-2 was not expressed in granulosa cells, however in that study no antigen retrieval was performed prior to the IHC for Tie-2, thus suggesting that the expression of Tie-2 in granulosa cells was not detected due to masking of the antigenic epitopes. To confirm the expression of Tie-2 in rat granulosa cells qRT-PCR was performed and Tie-2 was found to be expressed in granulosa cells. Tie-2 has not previously been shown to be expressed in the granulosa cells of rat follicles but is expressed in granulosa cells of primordial, primary and preantral follicles in the marmoset, and in antral follicles in the horse and sheep (Ellenberger et al., 2009, Chowdhury et al., 2010) suggesting that the expression detected in this thesis is genuine despite

disagreeing with published findings. Since the expression of Tie-2 had not been previously detected in rat granulosa cells it was concluded that the effects of Ang-1 on granulosa cells were promoted in a paracrine manner via signalling through the Tie-2 receptors in the thecal layer. However, the discovery that Tie-2 is expressed in granulosa cells suggests that the effects of Ang-1 on these cells are mediated directly.

Ang-1/Tie-2 signalling has been shown to play an important role in an anti-apoptotic system (Valable et al., 2003, Arai et al., 2004, Abramovich et al., 2006) and the inhibition of Ang-1 in rat ovaries *in vivo* promoted follicular atresia and reduced follicle number (Parborell et al., 2008). These findings suggest that Ang-1 may have an extravascular role in the ovary. This chapter investigated the role of Ang-1 in follicular atresia, in granulosa cell apoptosis and the mechanisms of Ang-1 action. Interestingly, treatment with Ang-1 at low doses resulted in the inhibition of activated caspase-3 expression whereas treatment with higher doses induced activated caspase-3 activity, suggesting that Ang-1 can have both a pro-survival and a pro-apoptotic effect on granulosa cells, in a dose dependent manner.

It has been shown that Ang-1 signalling via Tie-2 can activate Akt, resulting in the activation of pro-survival pathways and the suppression of pro-apoptotic pathways (Augustin et al., 2009). In addition, the treatment of HUVECs with Ang-1 resulted in the simultaneous activation of ERK1/2 and p38 (Harfouche et al., 2003). Therefore the effect of Ang-1 on key pro- and anti-apoptotic signalling pathways was investigated to further elucidate the mechanisms of Ang-1 action. Treatment of isolated granulosa cells with low doses of Ang-1 resulted in the up-regulation of



pERK1/2 and the inhibition of p38. Moreover, treatment with high doses of Ang-1 lead to the up-regulation of p38 showing that, dependent on the dose, Ang-1 can stimulate both pro-survival and pro-apoptotic pathways. This suggests that the overall effect on cell survival is determined by the level of Ang-1 stimulation.

As the receptor for Ang-1 is expressed on granulosa cells and the treatment of this cell type with Ang-1 results in the direct inhibition or stimulation of pro- and anti-apoptotic pathways it is likely that Ang-1 has a direct functional role in promoting granulosa cell survival, independent of its role in angiogenesis. To investigate the importance of the Tie receptors, Tie-1 and Tie-2, and also of Ang-2 knockdown experiments were performed to determine the effect on follicle health and angiogenesis when these factors are suppressed. Tie-1 has been shown to regulate the sensitivity of Tie-2 to Ang-1 activation and as Tie-1 can be cleaved by VEGF, the effect of Tie-1 knockdown on VEGF signalling was investigated. It was found that there was a reduction in VEGF expression after treatment with Ang-1, in a dose dependent manner. As VEGF would not be cleaving Tie-1 in these cells it is possible that the reduced expression is due to reduced demand for VEGF.

It would have been informative to determine whether this reduction in VEGF expression would result in reduced angiogenesis as if this was the case it would suggest that Tie-1 is required for the angiogenesis induced by Ang-1 treatment. Unfortunately it was not possible to perform these experiments during the course of this thesis. This chapter has also shown that the expression of Tie-2 and Ang-2 can

be suppressed at the RNA level however investigation into the downstream effects of this knockdown was also not possible during the course of this thesis.

In conclusion, this chapter has shown that Ang-1 is able to promote follicular angiogenesis. In addition it has been demonstrated that Tie-2 is expressed in rat granulosa cells, which suggests a functional role for Ang-1 in promoting granulosa cell survival. Treatment with Ang-1 at low doses triggers pro-survival pathways and at high doses induces pro-apoptotic pathways showing that Ang-1 can promote granulosa cell survival but can also induce apoptosis. These findings suggest that Ang-1 is important for follicle health and survival and this has implications for the improvement of culture systems for follicular development.

# **Chapter 7 2-Methoxyestradiol Inhibits Angiogenesis and Promotes Follicular Atresia *in vitro***

## **7 Chapter 7 2-Methoxyestradiol Inhibits Angiogenesis and Promotes Follicular Atresia *in vitro***

### **7.1 Introduction**

Angiogenesis is a key factor in many clinical conditions so the development of anti-angiogenic therapies is an area of great research interest. 2-methoxyestradiol (2-ME) is a naturally occurring derivative of 17 $\beta$ -estradiol that is synthesised in granulosa cells by hydroxylation at the 2-position and then subsequent catechol-O-methyltransferase mediated O-methylation (Shang et al., 2001, Thurston, 2002, Dahut et al., 2006). There is growing evidence that 2-ME has anti-tumour and anti-angiogenic activity (Fotsis et al., 1994, Pribluda et al., 2000, Ricker et al., 2004) and it has been demonstrated to directly inhibit proliferating endothelial cells and tumour cells. Although 2-ME is an oestrogen derivative it has low binding affinity for oestrogen receptors  $\alpha$  and  $\beta$ , and its anti-proliferative action is not mediated through an oestrogen-mediated pathway (LaVallee et al., 2002). The mechanisms of 2-ME induced inhibition of angiogenesis need further elucidation so this was investigated using the *in vitro* angiogenesis assay described in Chapter 3.

As the ovary expresses high levels of oestrogen it is likely that 2-ME is produced in the ovary and previous studies have shown that 2-ME is synthesised in ovarian follicles and is present in porcine follicular fluid, with the concentration increasing as folliculogenesis progresses (Basini et al., 2007, Salih et al., 2008). 2-ME has been shown to inhibit endothelial cell proliferation and to have anti-angiogenic activity in both *in vitro* and *in vivo* models of angiogenesis. Additionally it can inhibit mitosis in

granulosa cells suggesting that it could have anti-proliferative effects in non-vascular cell types (Shang et al., 2001). 2-ME can inhibit the production of VEGF and can also interact with HIF1  $\alpha$  suggesting that the anti-proliferative action of 2-ME could be due to negative regulation of the activity of VEGF via the inhibition of HIF1 $\alpha$  (Mabjeesh et al., 2003, Basini et al., 2007, Kim et al., 2010).

As 2-ME is present in ovarian follicles it has been speculated that it could have an extravascular role in the ovary, possibly in regulation of follicle development (Salih et al., 2008). 2-ME has been shown to induce apoptosis (Pribluda et al., 2000) and studies using immortalised granulosa cell lines have indicated that 2-ME may be able to induce apoptosis in granulosa cells. 2-ME has also been shown to activate the caspase cascade (Mooberry, 2003a, Mooberry, 2003b), induce p38 activation (LaVallee et al., 2003, Shimada et al., 2003) and inhibit Akt (Lin et al., 2007). Therefore it was hypothesised that 2-ME could be a negative regulator of cell survival due to the stimulation of pro-apoptotic mediators and/or the suppression of pro-survival pathways.

The mechanisms underlying the anti-proliferative action of 2-ME are still unclear so the potential mechanisms of action were investigated *in vitro*. In this chapter, the effect of 2-ME on follicular angiogenesis was investigated using the *in vitro* angiogenesis assay described in Chapter 3. The present study first determined the inhibitory effect of 2-ME on follicular angiogenesis and then investigated its effect on granulosa cell survival *in vitro*.

## 7.2 Materials and Methods

### 7.2.1 Follicle Culture

For all experiments, preantral/early antral follicles were cultured individually in flat-bottomed 12-well plates, embedded in the centre of a 20µl droplet of growth-factor reduced Matrigel. The Matrigel provides a three-dimensional extracellular matrix support for follicle growth and allows the vascular outgrowths to develop in three-dimensions, as they would *in vivo*. Follicles were incubated for 6 days in a sterile humidified air atmosphere with 5% CO<sub>2</sub> at 37°C in 600µl serum-free EBM-2 medium supplemented with BSA (0.1%), L-glutamine (3mM), penicillin (100 IU/ml), streptomycin (0.1mg/ml), transferrin (2.5µg/ml), selenium (4ng/ml), insulin (10ng/ml) and L-ascorbic acid, sodium salt (50µg/ml), with culture medium refreshed every 48h.

In order to test the effects of 2-ME, follicles were randomly placed into groups of 6 and cultured in control medium or in the presence of 0.01, 0.1, 1, 10µg/ml 2-ME (SB721 Batch 1A, Shimoda Biotech, Port Elizabeth, South Africa) and the experiment was carried out twice.

#### 7.2.1.1 Analysis of Angiogenic Sprouting

To assess the impact of the treatments on angiogenic sprouting, follicles were examined and photographed directly in the 12-well plates under an inverted light microscope, at x10 magnification, with Axiovision image capturing software on days 0 and 6 of the culture period. The total area of angiogenic sprouting for each follicle

was determined using Image Pro Plus software. This was done by drawing an area of interest around the outgrowths (but excluding the follicle), and the sum of the area of all sprouts was calculated by the software. The mean area of angiogenic sprouting for each treatment was then compared to the control.

## **7.2.2 Granulosa Cell Experiments**

### ***7.2.2.1 Isolation and culture of granulosa cells***

To investigate whether 2-ME can induce apoptosis in granulosa cells directly, isolated granulosa cells were cultured in 0.1ml medium containing  $1 \times 10^4$  cells/well. Following overnight preincubation in a sterile humidified air atmosphere with 5% CO<sub>2</sub> at 37°C, prewarmed 0.1ml medium containing with 0, 0.01, 0.1, 10 or 100µg/ml 2-ME was added to each well for 48h. All treatments were carried out in triplicate and experiments were repeated three times.

### ***7.2.2.2 Caspase-3 and -7 activity assay***

To measure caspase-3 and -7 activities in isolated granulosa cells, the Caspase-Glo 3/7 assay was performed according to the manufacturer's instructions, 48h after treating the cells (see Chapter 2). Treatments were carried out in triplicate and experiments were repeated three times to reduce the possible effects of biological variability. As serum can generate a background caspase activity signal, an additional two wells contained cell culture medium and carrier solution without any cells and TNFα was used as a positive control.

### **7.2.2.3 Western Blotting**

Protein was extracted from isolated granulosa cells as described previously (Chapter 2) and were resolved on 4–20% Tris-glycine gels and transferred onto a PVDF-fl membrane. Membranes were blocked for 1h at 25°C in Rockland Near Infra Red blocking buffer and incubated overnight at 4°C in blocking buffer containing rabbit anti-phospho-p42/44 ERK (1:1000), rabbit anti-phospho-p38 (1:500), HIF1 $\alpha$  (1:500) or VEGF (1:200) and  $\beta$ -actin (1:1000). After washing in PBS containing 0.5% Tween, membranes were incubated for 1h at 25°C in the dark in donkey anti goat IgG conjugated to IRDYE 800 and donkey anti rabbit IgG conjugated to Alexa Fluor 680 both at a dilution of 1:5000 in blocking buffer. Membranes were washed and proteins visualized and quantified using an Odyssey Infrared Imaging System. Sample loading was standardized to expression of  $\beta$ -actin.

### **7.2.3 Statistical Analyses**

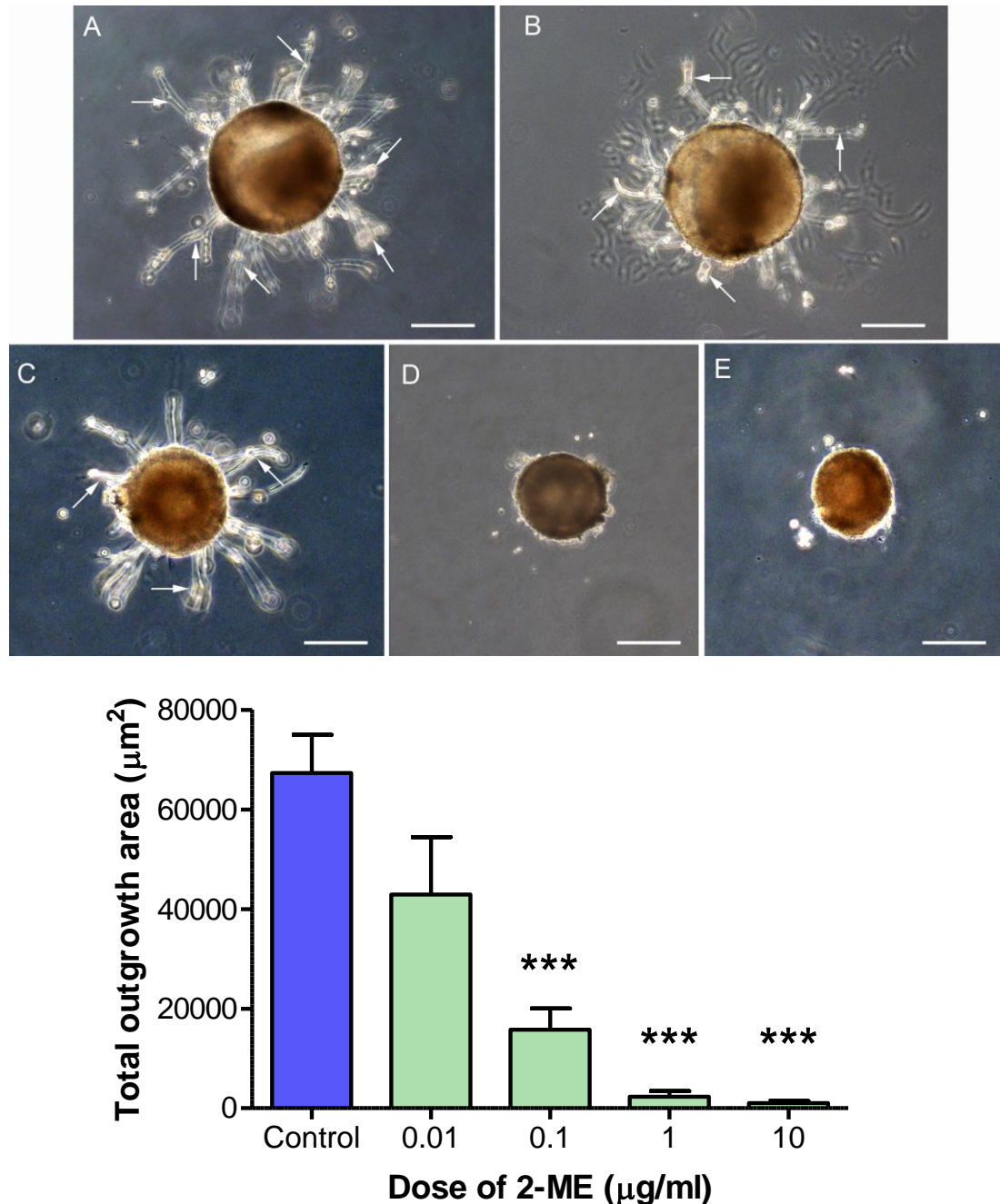
Data are presented as mean  $\pm$  SEM and all results were analysed statistically using Graph Pad Prism 5 software (Graph Pad Software). The results were analysed by means of a One-way ANOVA with a Bonferroni post-hoc test performed if the 95% confidence interval was reached to determine significance between all of the data groups. P values  $<0.05$  were accepted as statistically significant.



## 7.3 Results

### 7.3.1 Effect of 2-ME on angiogenesis *in vitro*

To investigate the effect of 2-ME on follicular angiogenesis, follicles were cultured in serum-free EBM-2 medium treated with 0, 0.01, 0.1, 1 and 10µg/ml 2-ME for six days. Treatment with 2-ME resulted in a significant inhibition of follicular angiogenesis, in a dose dependent manner, (Figure 2) ( $P < 0.001$ ), showing that 2-ME is able to inhibit angiogenesis in whole ovarian follicles *in vitro*.

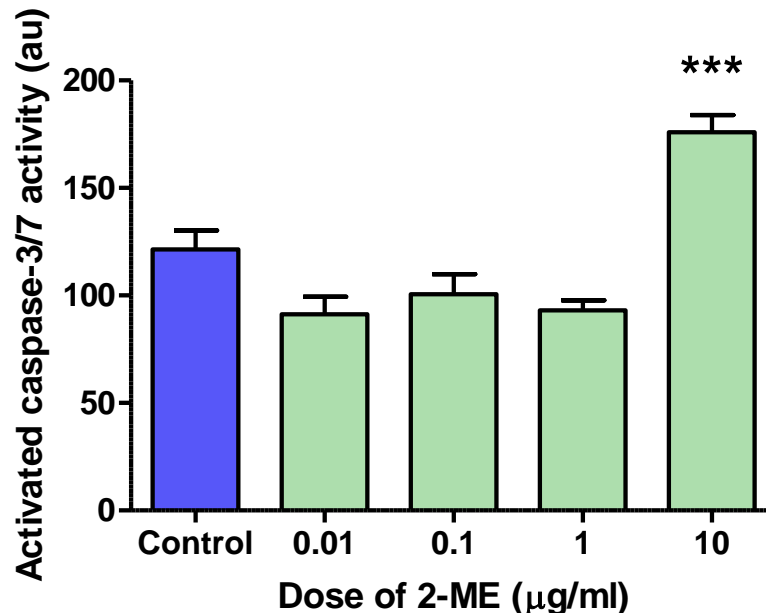


**Figure 7.1 Effect of 2-ME on angiogenesis**

To determine the effect of 2-ME on angiogenesis isolated preantral and early antral follicles ( $n=6$  per treatment) were cultured with varying doses of 2-ME. At the end of the culture period the area of the angiogenic outgrowths was measured using Image Pro-Plus software. Representative images show endothelial outgrowths from a control follicle (A) and follicles after treatment with 2-ME at  $0.01\mu\text{g/ml}$  (B),  $0.1\mu\text{g/ml}$  (C),  $1\mu\text{g/ml}$  (D) or  $10\mu\text{g/ml}$  (E). Results were quantified and analysed statistically by One-way ANOVA with a Bonferroni post-hoc test to compare the 2-ME treated follicles to the control follicles. Treatment with  $0.1$ ,  $1$  and  $10\mu\text{g/ml}$  2-ME resulted in a significant decrease in outgrowth area when compared to the control ( $***P<0.001$ ) (F). White arrows indicate the endothelial outgrowths. Scale bars represent  $200\mu\text{m}$ . Experiments were repeated in triplicate.

### 7.3.2 Effect of 2-ME on granulosa cell apoptosis *in vitro*

To investigate the effect of 2-ME on follicular apoptosis, isolated granulosa cells were cultured with 0, 0.01, 0.1, 1 and 10 µg/ml 2-ME for 48h followed by quantification of apoptosis using a luminescent caspase-3/7 assay. There was a significant increase in activated caspase-3/7 activity in granulosa cells treated with the highest dose of 2-ME ( $P < 0.001$ ) (Figure 3), showing that 2-ME does act directly on granulosa cells to stimulate apoptosis.



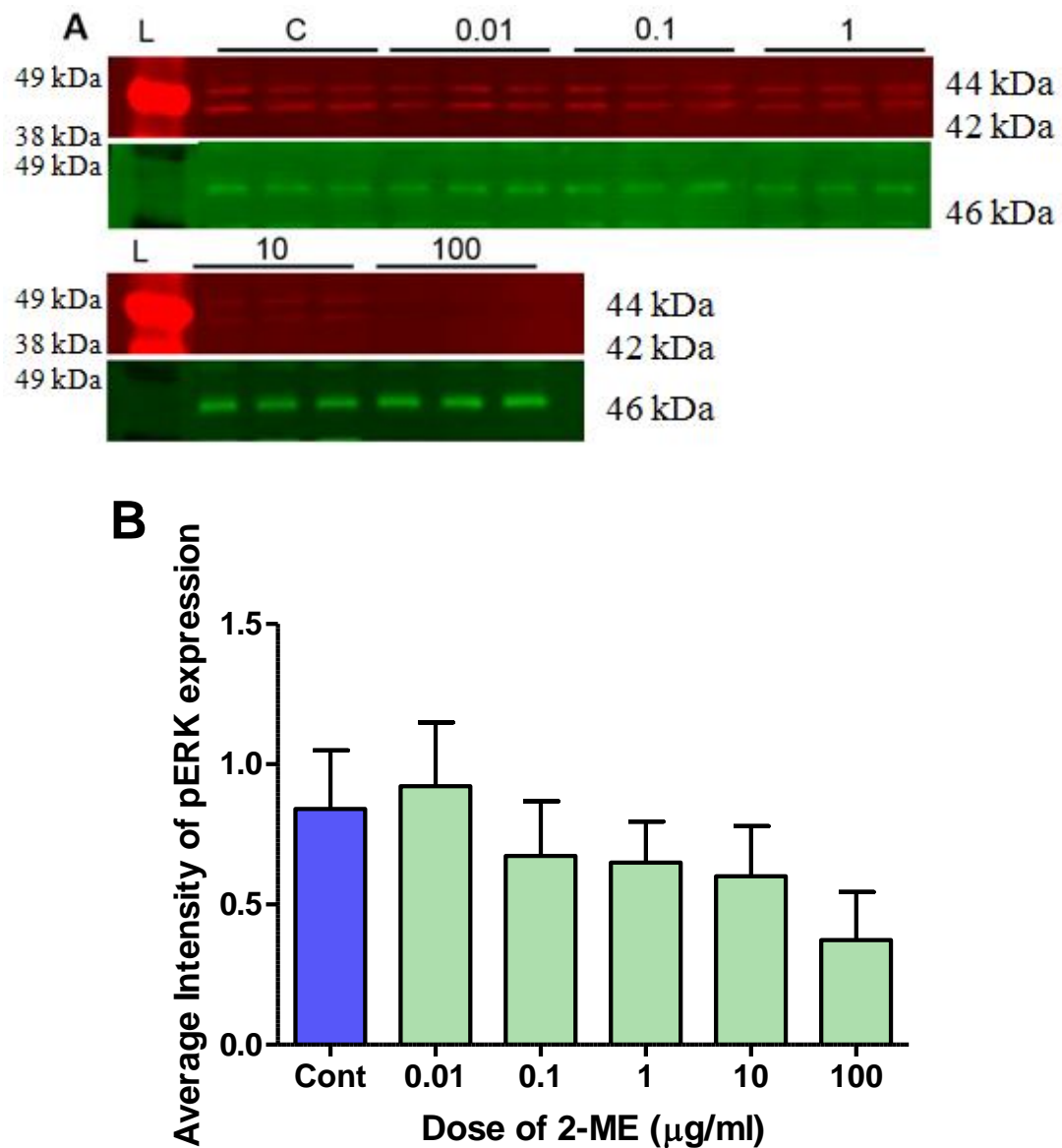
**Figure 7.2 Effect of 2-ME on granulosa cell apoptosis**

To determine the effect of 2-ME on apoptosis granulosa cells were isolated from preantral and early antral follicles and they were cultured with varying doses of 2-ME ( $n=4$  per treatment). At the end of the culture period these granulosa cells were analysed for expression of activated caspase-3/7 activity using a luminescent caspase activity assay, as an indicator of follicle health. A One-way ANOVA with a Bonferroni post-hoc test was performed on the results to compare the 2-ME treated granulosa cells to the control granulosa cells and there is a significant increase in activated caspase-3 expression after treatment with 10 µg/ml 2-ME when compared to the control ( $***P < 0.001$ ). Luminescence is given in arbitrary units (au). Experiments were repeated in triplicate.

### **7.3.3 Mechanisms of the pro-apoptotic effect of 2-ME**

#### ***7.3.3.1 Quantification of pERK expression by western blotting***

As treatment with 2-ME can induce apoptosis further investigations were carried out to establish the mechanisms by which 2-ME induces cell death. Phospho-ERK (pERK) is an important pro-survival factor so the effect of 2-ME on the expression of pERK was investigated. Isolated granulosa cells were cultured with 0, 0.01, 0.1 10 and 100µg/ml of 2-ME for 48h followed by quantification of the expression of pERK by western blotting (Figure 7.3A). The results showed that there was a trend of decreasing expression of pERK (Figure 7.3B), suggesting that 2-ME could induce apoptosis through the suppression of pro-survival mediators.

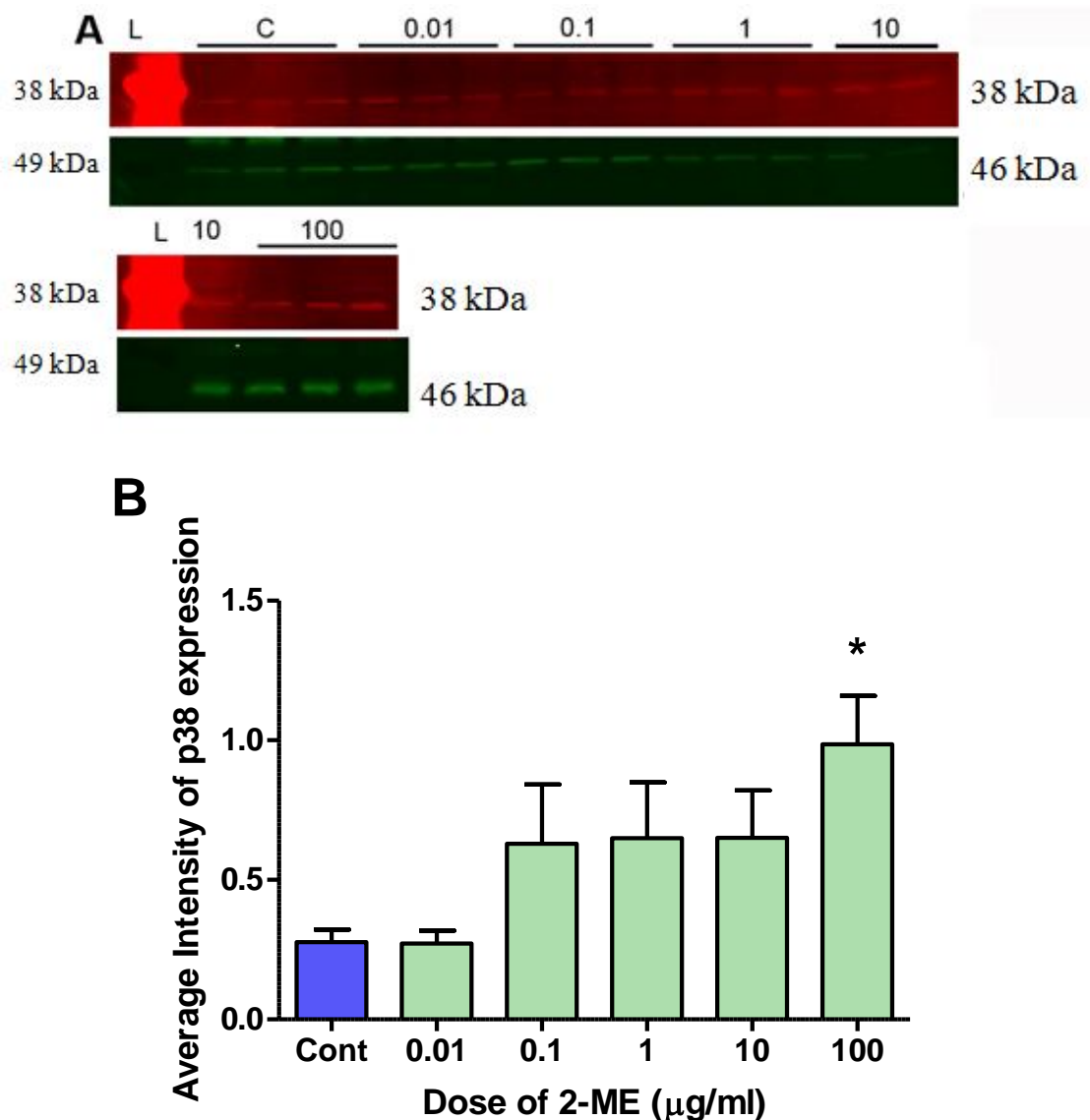


**Figure 7.3 Levels of pERK expression after treatment with 2-ME**

Isolated granulosa cells were cultured with varying doses of 2-ME ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed. To try to determine a potential mechanism of apoptosis induction the level of pERK expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with 2-ME. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA to compare the 2-ME treated granulosa cells to the control granulosa cells. The results show that there was a trend of decreasing expression of pERK after treatment with 2-ME; however this was not statistically significant. Experiments were repeated in triplicate.

#### **7.3.3.2 Quantification of p38 expression by western blotting**

The expression of pro-apoptotic factor p38 was then investigated to further elucidate the mechanisms of apoptosis induction. To investigate this isolated granulosa cells were treated with 0, 0.01, 0.1 10 and 100µg/ml of 2-ME for 48h followed by quantification of the expression of p38 by western blotting (Figure 7.4A). The results showed that there was a significant increase ( $P<0.05$ ) (Figure 7.4B) in the expression of p38 after treatment with 2-ME. As 2-ME stimulates both activated caspase-3 and p38 it is likely that 2-ME induces apoptosis by activating pro-apoptotic pathways.



**Figure 7.4 Levels of p38 expression after treatment with 2-ME**

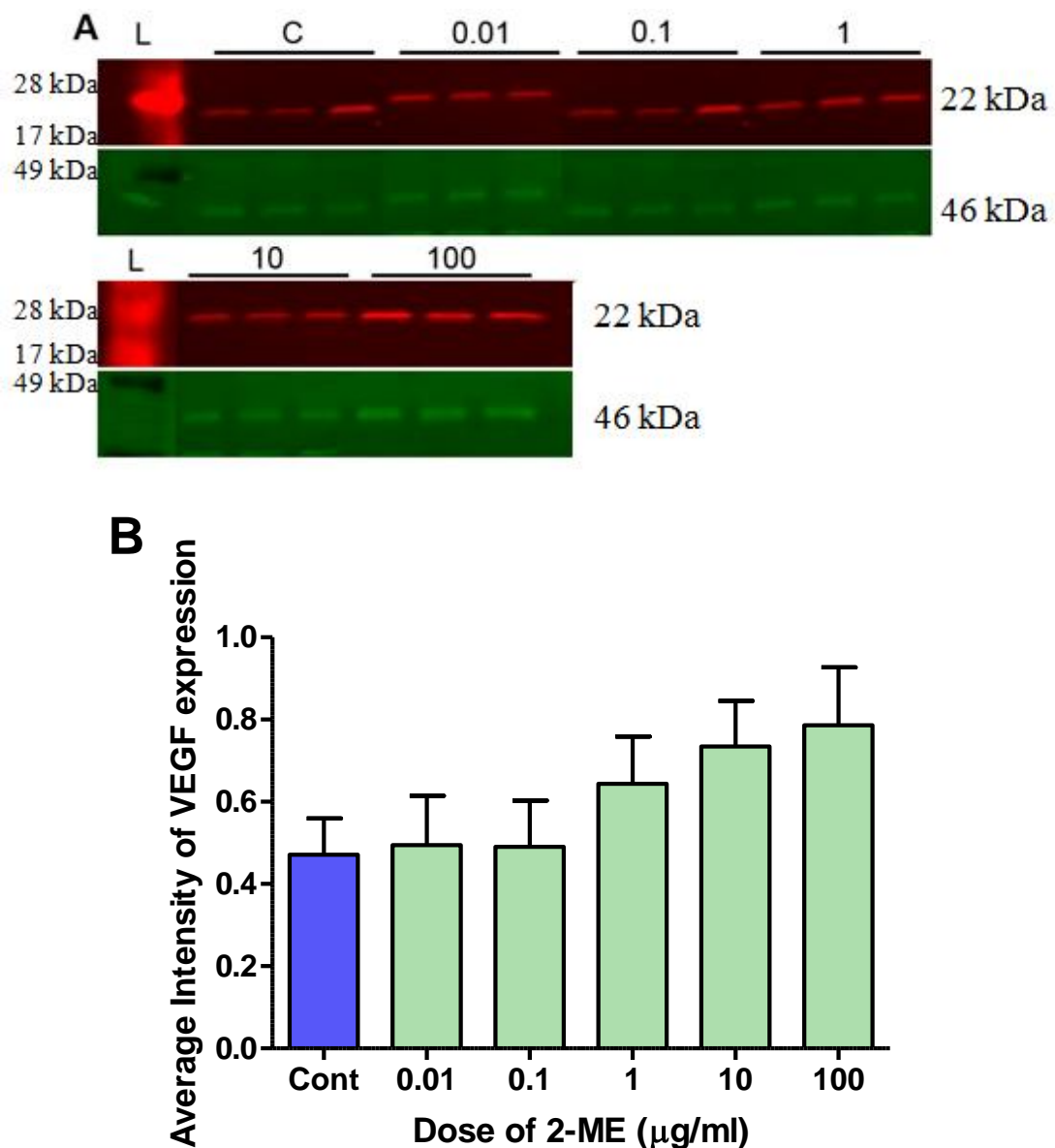
Isolated granulosa cells were cultured with varying doses of 2-ME ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed. To try to determine a potential mechanism of apoptosis induction the level of p38 expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with 2-ME. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA with a Bonferroni post-hoc test to compare the 2-ME treated granulosa cells to the control granulosa cells. The results show that there is a significant increase in the expression of p38 after treatment with 100 µg/ml 2-ME when compared to the control (\* $P<0.05$ ). Experiments were repeated in triplicate.

### **7.3.4 Mechanisms of the anti-angiogenesis**

#### ***7.3.4.1 Quantification of VEGF expression by western blotting***

2-ME has been shown to inhibit angiogenesis and it has previously been shown that 2-ME can act on VEGF and suppress its expression (Basini et al., 2007). Therefore it was hypothesised that 2-ME could be inhibiting angiogenesis through the suppression of VEGF. To investigate this isolated granulosa cells were treated with 0, 0.01, 0.1 1, 10 and 100µg/ml of 2-ME for 48h followed by quantification of the expression of VEGF by western blotting (Figure 7.5A). The results showed that there was no difference in the expression of VEGF after treatment with 2-ME (Figure 7.5B).



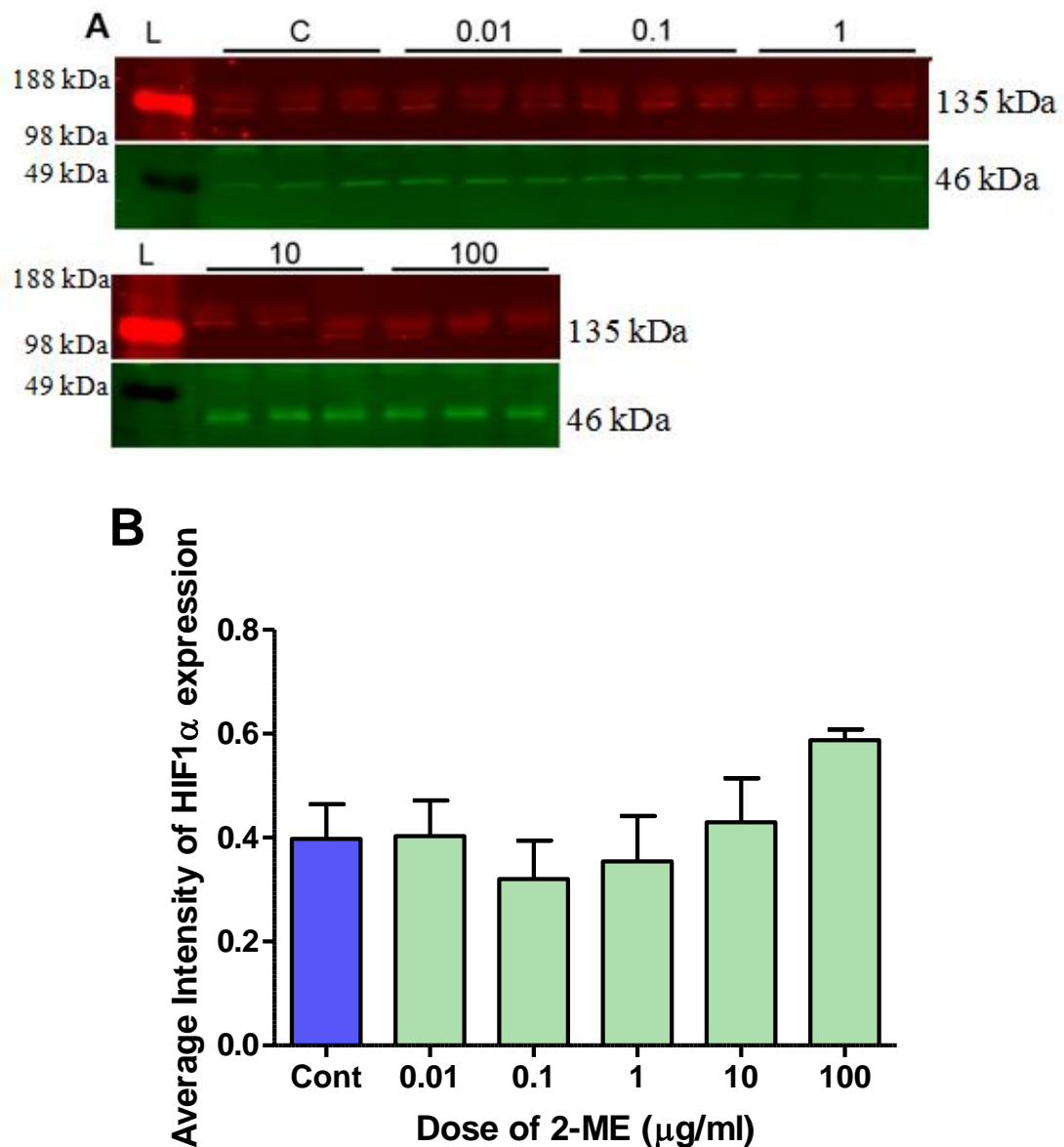


**Figure 7.5 Levels of VEGF expression after treatment with 2-ME**

Isolated granulosa cells were cultured with varying doses of 2-ME ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed. To try to determine a potential mechanism of anti-angiogenesis the level of vascular endothelial growth factor (VEGF) expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with 2-ME. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA to compare the 2-ME treated granulosa cells to the control granulosa cells. There was no significant difference in the expression of VEGF after treatment with 2-ME. Experiments were repeated in triplicate.

#### **7.3.4.2 Quantification of HIF1 $\alpha$ expression by western blotting**

Hypoxia is known to stimulate angiogenesis and as 2-ME inhibits angiogenesis, the effect of 2-ME on the expression of HIF1 $\alpha$  was investigated (Kim et al., 2010). To investigate this isolated granulosa cells were treated with 0, 0.01, 0.1 10 and 100 $\mu$ g/ml of 2-ME for 48h followed by quantification of the expression of HIF1 $\alpha$  by western blotting (Figure 7.6A). The results showed that there was no difference in the expression of HIF1 $\alpha$  after treatment with 2-ME (Figure 7.6B).



**Figure 7.6 Levels of HIF1 $\alpha$  expression after treatment with 2-ME**

Isolated granulosa cells were cultured with varying doses of 2-ME ( $n=3$  per treatment) and then protein was extracted to allow western blotting to be performed. To try to determine a potential mechanism of anti-angiogenesis the level of hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) expression was measured by western blotting (A). L – size ladder. Gels were laid out with the size marker on the left-hand side of the gel, the control samples and then the samples treated with 2-ME. The control samples were used on each gel so that a direct comparison to the expression in the control, on that gel, could be made. The size of the bands of interest has been shown on the right of the figure with the size marker bands on the left. These data were quantified (B) and analysed statistically by One-way ANOVA to compare the 2-ME treated granulosa cells to the control granulosa cells. There was no significant difference in the expression of HIF1 $\alpha$  after treatment with 2-ME. Experiments were repeated in triplicate.

## 7.4 Discussion

In this chapter the *in vitro* angiogenesis assay described in Chapter 3 was utilised to demonstrate that 2-ME is a potent inhibitor of follicular angiogenesis. Furthermore, it has been shown that 2-ME promotes follicular atresia *in vitro* by directly inducing apoptosis of granulosa cells, suggesting that it has a functional role in regulating granulosa cell survival *in vitro*.

2-ME has been shown to have anti-proliferative activity in endothelial cells and therefore has been suggested as an anti-angiogenic agent. 2-ME is produced in granulosa cells and is present in follicular fluid in ovarian follicles (Basini et al., 2007, Salih et al., 2008), suggesting that 2-ME could play a role in regulating follicular angiogenesis. This chapter has demonstrated that 2-ME inhibits follicular angiogenesis in a dose dependent manner. The mechanisms of 2-ME-induced inhibition of angiogenesis were investigated by quantifying the levels of some of the factors involved in the angiogenic cascade.

Previous studies have suggested that the anti-proliferative effects of 2-ME may be due to the inhibition of HIF1 $\alpha$  expression (Kim et al., 2010). 2-ME inhibits the activation of HIF1 $\alpha$  (Mabjeesh et al., 2003, Ricker et al., 2004, Lu et al., 2010) *in vitro* and has been used to inhibit HIF1 $\alpha$  in tumour cells, indicating that 2-ME can inhibit hypoxia. The up-regulation of HIF1 $\alpha$  expression results in increased expression of VEGF and the induction of angiogenesis so the effect of 2-ME treatment on the expression of HIF1 $\alpha$  and VEGF was investigated to elucidate the mechanisms of angiogenesis inhibition by 2-ME. However, there was no difference

in the expression of HIF1 $\alpha$  or VEGF protein following treatment with 2-ME, suggesting that the regulation of angiogenesis by 2-ME in the ovary involves the interaction of many factors and different pathways, which require further investigation.

Treatment with 2-ME increases atresia via the stimulation of activated caspase-3, one of the main molecules involved in the induction of apoptosis, so it is likely that 2-ME also regulates the expression of other pro- or anti-apoptotic mediators. It was anticipated that 2-ME could have an effect on the expression of pERK and/or p38 (LaVallee et al., 2003, Mooberry, 2003a, Mooberry, 2003b, Shimada et al., 2003, Lunn et al., 2002, Lin et al., 2007) as they are key factors involved in anti- and pro-apoptotic pathways, respectively, that either lead to the inhibition or induction of the caspase cascade. The ERK/MAPK pathways play an important role in the regulation of apoptosis so the expression of pERK and p38 after treatment with 2-ME was quantified to clarify the mechanisms of apoptosis induction by 2-ME. This chapter has shown that there is a trend of decreasing pERK expression and that there is a significant increase in p38 expression. These results agree with other studies which have shown that 2-ME can induce apoptosis through the activation of p38 (Lunn et al., 2002), suggesting that 2-ME induces follicular apoptosis through the stimulation of pro-apoptotic pathways. However, it may also inhibit pro-survival mediators.

Since 2-ME can inhibit cellular proliferation and induce apoptosis it has been suggested as a potential therapeutic agent for cancer treatment, especially in tumours of a reproductive nature (Lunn et al., 2002, Dahut et al., 2006). As a result 2-ME has

been used in several clinical trials but has been shown to have poor bioavailability and potency (Dahut et al., 2006, Adams et al., 2004). It will therefore be necessary to improve its pharmacokinetic properties before it can effectively be used in the clinic. It has also been suggested that 2-ME could be beneficial in the treatment of PCOS; however the studies that indicated this were performed in immortalised granulosa cells (Salih et al., 2008), making it difficult to apply these results to the clinical situation. Overall the investigations into the effects of 2-ME have suggested that it could have many clinical benefits but additional studies are required to improve its therapeutic potential.

In conclusion, this chapter has demonstrated that 2-ME inhibits angiogenesis in the ovarian follicle and has a functional role in the induction of follicular atresia. It has been shown that 2-ME directly promotes apoptosis in primary granulosa cells, indicating an extravascular role for 2-ME during follicular development. 2-ME is a promising candidate for clinical use in the treatment of abnormal angiogenesis and further studies will hopefully confirm the potential of 2-ME as a therapeutic for reproductive disorders.

## **Chapter 8 General Discussion**

## 8 Chapter 8 General discussion

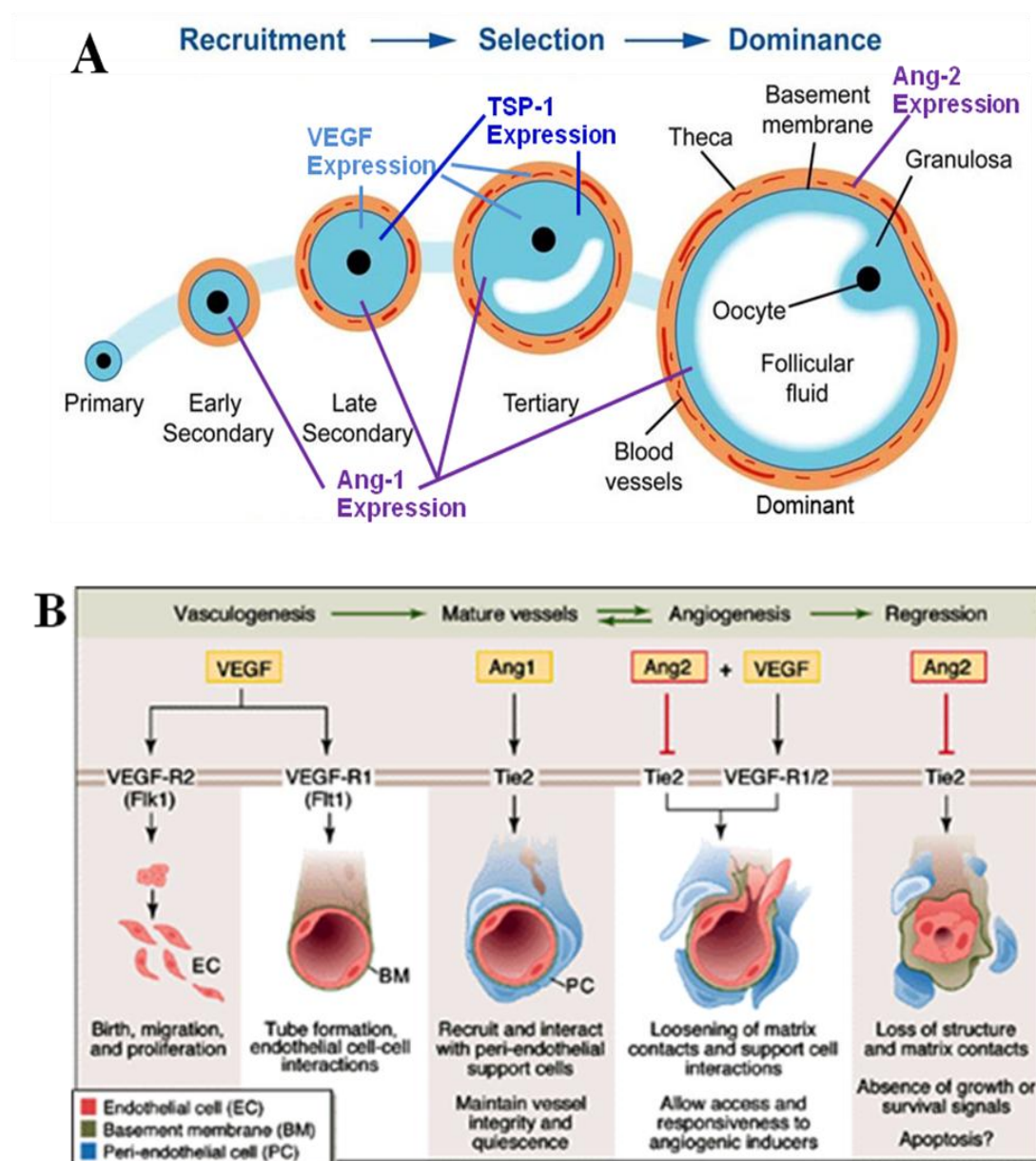
This thesis has developed a novel *in vitro* angiogenesis assay for the study of follicular angiogenesis and it has been utilised to investigate the role of various pro- and anti-angiogenic factors in ovarian follicular angiogenesis *in vitro* in the rat and *in vivo* in the marmoset. The possibility of an extravascular role for these factors was also investigated using the *in vitro* assay and this chapter summarises the main findings of the thesis. The clinical implications of these findings and future research directions are also considered.

### 8.1 The findings of the thesis

The main findings of this thesis were:

- Ovarian follicles can produce angiogenic outgrowths in *in vitro* culture
- TSP-1 inhibits angiogenesis both *in vitro* and *in vivo*
- TSP-1 induces follicular atresia both *in vitro* and *in vivo*
- Ang-1 plays a role in regulating follicle health and survival
- The receptor for Ang-1 has been shown to be expressed in rat granulosa cells
- 2-ME can inhibit angiogenesis and increase granulosa cell apoptosis
- Factors involved in angiogenesis also have extravascular roles within the ovary, often in regulating follicle survival





**Figure 8.1** Diagram showing the main expression of the various factors used in this thesis

This diagram summarises the stages of follicular development when the main pro- or anti-angiogenic factors discussed in this thesis are expressed (A) and also shows the action and interaction of these factors and their receptors (B). Fig 8.1B is adapted from (Hanahan, 1997).

## **8.2 The *in vitro* follicle culture system as an angiogenesis model**

There are many *in vitro* assays currently available for the study of angiogenesis however they all have limitations. Furthermore, ovarian follicles are routinely cultured to investigate factors that affect follicle health and development and are relatively easy to handle and are widely available. Follicles grow well in *in vitro* culture and immature rat follicles develop in a short time frame indicating that an assay based on ovarian follicles could produce results quickly. The female reproductive system is unique as no other healthy adult tissues undergo spontaneous angiogenesis. Therefore in this thesis the natural angiogenic properties of ovarian follicles were utilised to provide a novel angiogenesis assay.

Preantral and early antral follicles have undergone endothelial cell recruitment from the ovarian stroma and follicles at these stages can be easily isolated from immature rats. This thesis has demonstrated that isolated follicles can produce angiogenic outgrowths when cultured in the appropriate conditions and that these outgrowths are endothelial in nature and are surrounded by pericytes, suggesting that they are representative of the vessels that develop *in vivo*. The cultured follicles produced a dense network of blood vessels quickly, with blood vessels apparent after two days and a network of vessels established between 4-6 days of culture. The vessels could then be quickly and accurately quantified, allowing results to be determined in a short time period. Furthermore, the large numbers of preantral and early antral follicles present in the ovaries of immature rats allows several doses of a compound to be investigated in one experiment whilst keeping numbers high and the number of

animals used low. The evaluation of this culture system and its ease of use suggest that this is a valid method for the study of follicular angiogenesis and that it could be a useful tool for the investigation of angiogenesis. Additionally, as both physiological and pathological angiogenesis take place by very similar mechanisms and are regulated by many of the same factors it is likely that an angiogenesis model that allows detailed investigation of physiological angiogenesis and the regulation of the factors involved in it would be beneficial in improving the understanding of both physiological angiogenesis and pathological angiogenesis. The regulation of the factors involved in physiological angiogenesis is altered in pathological angiogenesis and improving the understanding of the importance of these factors in angiogenesis could help develop treatments for pathological angiogenesis. Furthermore, the angiogenesis utilised in the *in vitro* follicle culture assay occurs naturally so there is no need to induce disease to study the angiogenic process. Therefore, with all of these facts in mind, the *in vitro* follicle culture assay developed in this thesis is a good model for angiogenesis.

### **8.2.1 Limitations of the model**

Although this angiogenesis assay has provided new insight into follicular angiogenesis, it does have its limitations. The use of EBM-2 medium is necessary for the development of angiogenic outgrowths yet it does not provide optimum conditions for follicle health. Traditionally follicles are cultured in media such as  $\alpha$ MEM, McCoys or Waymouths, which allow the follicles to grow and develop during culture, but these media types do not allow the development of blood vessels. Therefore the conditions that would be optimal for follicle development are not ideal

for this assay. Substantial efforts were made during the course of this PhD to optimise the culture conditions to allow blood vessel development whilst still maintaining the health of the follicles, yet it was not possible to achieve optimal follicle health for long periods of time, alongside blood vessel development.

### **8.3 The *in vivo* model of angiogenesis and follicle development**

The *in vivo* investigation of angiogenesis provides a more comprehensive look at the role of factors in regulating angiogenesis and also allows a whole animal view of the effects of compounds of interest. The marmoset model of ovarian angiogenesis is well established and has been described in great detail by our group (Fraser et al., 2000, Fraser et al., 2006, Fraser and Duncan, 2009). This model has many advantages over other *in vivo* angiogenesis assays as it utilises and manipulates the natural angiogenic properties of the ovary and the angiogenesis that occurs is both natural and physiological. Additionally, the marmoset ovary contains numerous growing follicles at all stages of the cycle, allowing the effects on angiogenesis across the cycle to be seen in each ovary.

The marmoset is commonly used in biomedical research, especially in reproductive research, due to their genetic similarity to humans, ease of handling, high reproduction rate (Torii et al., 1996) and their small size (approximately 350g) which makes them ideal for use in *in vivo* studies as this allows the use of small amounts of the compound of interest. The marmoset ovarian cycle lasts for approximately 28

days (Harding et al., 1982, Hearn, 1983) and it can be easily manipulated. Our in-house marmoset facility provides numerous healthy, cycling marmosets thus allowing terminal studies to be carried out so that tissues of interest can be isolated and collected for analysis by immunohistochemistry and *in situ* hybridisation. In addition, the ability to synchronise the ovarian cycle through the induction of luteolysis after injection of prostaglandin enables the study of precise stages in the ovarian cycle (Summers et al., 1985, Duncan et al., 1998, Gilchrist et al., 2001). The development of ovarian vasculature has been described in great detail in the marmoset (Wulff et al., 2001a, Wulff et al., 2002) so this, along with the other factors mentioned, makes the marmoset a good model for the *in vivo* investigation of angiogenesis. The studies in this thesis utilised the marmoset monkey as an experimental model in which to investigate the role of ABT-898 in regulating ovarian follicular angiogenesis and development *in vivo*.

### 8.3.1 Limitations of the model

Although the marmoset has a reproductive cycle and hormonal profile similar to that in the human, the model does have limitations. The marmoset is a multi-ovulatory species that has a longer luteal phase than that in humans and does not menstruate. Old World primates have a reproductive cycle that is more similar to humans than that of New World primates and they also menstruate. However, the invasiveness of *in vivo* studies severely limits the use of higher primates in research.

In the *in vivo* studies carried out during this thesis, assays were performed to determine progesterone concentration but it would have been informative to evaluate

the function of the preovulatory follicles by determining changes in levels of plasma estradiol. However, no reliable assay for detecting changes in estradiol in the marmoset is available as detecting subtle differences in estradiol in marmosets may be confounded by high levels of circulating estrone (unpublished observations). Additionally, due to their small size, only a small volume of blood can be taken from the marmoset at each sampling interval, making it challenging to assay for multiple hormones.

#### **8.4 The importance of angiogenic factors in follicular development**

Angiogenesis plays an important role in follicular development as a blood supply is necessary for follicle maturation to occur. Angiogenesis must take place to allow the developing follicles to be supplied with gases, nutrients and hormones and a sufficient blood supply also allows endocrine, paracrine and autocrine regulation of follicular development to occur. Therefore it is an essential part of follicle development. Many of the receptors for factors that are involved in the regulation of angiogenesis are expressed within the ovary in non-vascular cells which has lead to the suggestion that they could have extravascular roles within the ovary.

Although the main role of these factors is in regulating angiogenesis many of these factors, such as VEGF, TSP-1 and Ang-1, have been shown to act on non-vascular cells within the ovary. VEGF is the key factor involved in promoting angiogenesis and it has been demonstrated that VEGF has a crucial role in angiogenesis, which has

a role in ovarian function (Geva and Jaffe, 2000, Danforth et al., 2003, Zimmermann et al., 2003, Abramovich et al., 2006). Studies in the ovary have shown that when VEGF expression is suppressed follicles do not undergo follicle development and ovulation is blocked, showing the vital role VEGF has in both ovarian angiogenesis and ovarian function (Wulff et al., 2001a, Wulff et al., 2001b, Fraser et al., 2005a, Fraser et al., 2005b, Fraser et al., 2006, Taylor et al., 2007). It has also been shown that VEGF can have a cytoprotective effect within the ovary and that inhibition of VEGF results in increased apoptosis (Greenaway et al., 2004), indicating that VEGF has non-vascular effects within the ovary. Additionally, treatment with VEGF increases the activation of ERK1/2 whilst the inhibition of VEGF suppresses the stimulation of ERK1/2 (Doyle et al., 2010); supporting the suggestion that VEGF has non-vascular effects in the ovary in regulating granulosa cell function.

The studies in this thesis have demonstrated that TSP-1, Ang-1 and 2-ME can all have extravascular actions within the ovary, in regulating cell survival mediators in granulosa cells. TSP-1 and 2-ME stimulate granulosa cell apoptosis through the up-regulation of pro-apoptotic mediators including p38, indicating that anti-angiogenic factors could have a role in clearing follicles that are not selected for development from the ovary through the stimulation of similar pro-apoptotic pathways. The pro-angiogenic factor Ang-1 stimulates the production of the pro-survival mediator pERK1/2 and reduces the expression of pro-apoptotic factors suggesting that it could improve follicle health and survival. Autocrine and paracrine signalling play an important role in regulating follicle development and several of the factors that regulate follicle development, such as activin and inhibin, are produced by the

granulosa cells themselves (Knight and Glister, 2006, Chedrese et al., 2009, Hillier, 2009). These factors have also been shown to stimulate pro-survival mediators, including ERK1/2 signalling (Wang and Tsang, 2007), through autocrine signalling. Therefore it is likely that the regulation of granulosa cell survival observed after treatment with the factors used in this thesis is through autocrine signalling in the granulosa cells.

Overall, this thesis has shown that a range of angiogenic factors have extravascular roles within the ovary and these effects involve the regulation of cell survival mediators. The discovery that angiogenic factors can have extravascular roles within the ovary suggests that they could have clinical applications outside of treating aberrant angiogenesis. Since factors such as Ang-1 may have a functional role in promoting follicular growth and survival, they could be novel factors for use in improving culture conditions for techniques such as *in vitro* maturation (IVM) protocols for the treatment of infertility.

## **8.5 Clinical implications of the findings of the thesis**

Angiogenesis is a tightly regulated process and a disturbance in the balance of pro- and anti-angiogenic factors that regulate it can result in many clinical conditions. Excess angiogenesis is associated with tumour growth, aggravation of inflammatory disorders and reproductive pathologies such as PCOS and endometriosis. Therefore the elucidation of the regulation of angiogenesis is important for the development of new therapeutics for disorders associated with aberrant angiogenesis such as cancer,



coronary artery disease, PCOS and endometriosis (Folkman, 2001, Kerbel and Folkman, 2002, Carmeliet, 2004). In addition insufficient angiogenesis is involved in a number of clinical conditions including increased incidence of pre-eclampsia (Levine et al., 2004) and many age related diseases (Carmeliet, 2004).

The detailed understanding of the factors involved in regulating angiogenesis is of great importance and although there are many assays available for angiogenesis research they all have limitations. The majority of existing *in vitro* assays are not representative of *in vivo* angiogenesis and the existing *in vivo* assays are often expensive and very technically demanding. Therefore, the development of an *in vitro* assay that would allow investigation into the roles of the factors involved in regulating angiogenesis and that would be more representative of angiogenesis *in vivo* would be of benefit. During the course of this thesis a novel *in vitro* assay has been developed for the investigation of follicular angiogenesis. The blood vessels that are produced in this assay develop from the endothelial cells present in the thecal layer of isolated preantral and early antral follicles. These blood vessels develop in three-dimensions around the follicle and are surrounded by supporting pericytes, as *in vivo*. These results suggest that the vessels that develop in this assay are representative of physiological follicular angiogenesis. Therefore, this assay could be beneficial in elucidating the role of factors involved in the regulation of follicular angiogenesis and could also be utilised to evaluate the effect of various compounds on follicular angiogenesis.

PCOS is the most common disorder in women of reproductive age (Franks, 1995) and is characterised by aberrant angiogenesis, hyperandrogenism and the accumulation of small anovulatory antral follicles with reduced follicular atresia (Franks et al., 2000, Agrawal et al., 2002). The existing treatments for PCOS are limited and are mainly aimed at controlling the symptoms of the disease. There are a few treatments, such as laser ablation, that results in a short period where ovarian cycles become ovulatory (Homburg, 2003, 2008) but there is currently no therapy that targets both the abnormal ovarian angiogenesis and the accumulated antral follicles. Treatment with TSP-1 and the thrombospondin mimetic ABT-898 inhibits follicular angiogenesis and also induces apoptosis of preantral and early antral follicles suggesting that it may be a potential treatment for PCOS as it could have a dual role in targeting the increased angiogenesis and inducing atresia of accumulated antral follicles without affecting preovulatory follicle development and ovulation.

Targeting angiogenesis may be an effective treatment for PCOS as it is characterised by increased angiogenesis and decreased expression of anti-angiogenic factors. Additionally, angiogenesis is required for follicle growth and development to the antral stage and in PCOS follicles halt development and accumulate in the ovaries. Therefore the inhibition of angiogenesis could prevent the development of follicles to the antral stage, reducing the accumulation of non-growing antral follicles. It has also been shown that the common PCOS treatment metformin attenuates angiogenesis, suggesting that the inhibition of angiogenesis can be beneficial in the treatment of PCOS. Overall, the inhibition of angiogenesis in PCOS should have beneficial results on the reproductive effects of the disease and could lead to periods of ovulation and

fertility for women suffering from this condition. However, PCOS has many wide-ranging effects and altering angiogenesis is unlikely to treat the wider effects of this condition.

Tumour development is dependent on the stimulation of new blood vessel growth from the pre-existing vasculature (Folkman et al., 1971, McDougall et al., 2006) as this allows them to be supplied with nutrients and gases. Therefore, the development of anti-angiogenic agents is a focus of therapeutics for a number of angiogenesis-related disorders. Anti-angiogenic agents deprive tumour cells of the nutrients, gases and survival factors that are required for their survival, resulting in the death of the tumour cells. There are many anti-angiogenic compounds under development (Kerbel and Folkman, 2002) that are undergoing various stages of clinical trials and have a variety of actions, including inhibiting the action of pro-angiogenic factors and inhibiting endothelial cell proliferation, migration and survival. Bevacizumab (Avastin<sup>TM</sup>), a humanised monoclonal antibody directed at VEGF, is the most advanced in clinical development (Ferrara, 2004). Bevacizumab was approved for use in metastatic cancer by the US Food and Drug Administration in 2004 (Huang et al., 2009) and has now been approved for use in a variety of cancers (Mancuso and Sternberg, 2006, Bossung and Harbeck, 2010, Chamberlain, 2010, Summers et al., 2010). In addition Aflibercept (Regeneron Pharmaceuticals), a recombinant chimeric protein comprising portions of the extracellular domains of the human VEGFR1 and VEGFR2 expressed in sequence with the Fc portion of human Ig (Holash et al., 2002), is currently in clinical trials and has shown promising results (Dixon et al., 2009, Tew et al., 2009, Twardowski et al., 2010).

Therapeutics have been designed to mimic the anti-angiogenic activity of TSP-1 and they are usually designed to mimic the activity of the TSR region. The potency of a first-generation mimetic of TSP-1, ABT-510, was found to be insufficient in human clinical trials so a second generation mimetic of the anti-angiogenic activity of TSP-1, ABT-898, has been developed. ABT-898 is thus expected to have greater efficacy than the other available TSP-1-mimetic peptides. ABT-898 has shown promising results in the *in vitro* and *in vivo* studies performed during the course of this PhD and will hopefully proceed to clinical trials after further investigations.

There are many assisted reproduction techniques (ART) available for people with impaired fertility and the majority of them rely on the *in vitro* maturation of follicles. The health of the follicles during this process determines the success of ART so the discovery of novel factors that can improve the survival of follicles *in vitro* could be of benefit. The studies carried out during this thesis have shown that various angiogenic factors, such as Ang-1, can act on non-vascular cells within the ovary to improve follicle survival. Ang-1 has been shown to stimulate the expression of pERK and inhibit the expression of caspase-3 in granulosa cells, indicating that it plays a role in promoting cell survival. Many studies have indicated that Ang-1 may have a role in inhibiting apoptosis and the finding that Ang-1 can improve follicle and granulosa cell health *in vitro* suggests that it could be useful in improving conditions for *in vitro* follicle development culture systems.

## 8.6 Suggestions for further study

During the course of this thesis a novel *in vitro* angiogenesis assay was developed to provide an assay that would allow the manipulation of follicular angiogenesis and would be representative of physiological angiogenesis. This assay could provide a method for mechanistic studies which would be a valuable tool in angiogenesis research and could be of benefit in elucidating the regulation of follicular angiogenesis. Additionally it could be used to investigate the effects of new compounds on follicular angiogenesis, allowing the screening of compounds of interest prior to investigation *in vivo*. This thesis has found that several angiogenic factors have extravascular roles within the ovary so the use of this *in vitro* angiogenesis assay would also enable the investigation into roles for these factors in follicle development and survival.

The follicle culture system developed during this thesis could also be used to develop a directional model of growth. This could be done by using a transwell assay where endothelial cells are plated on one side of a porous membrane, and a solution containing the potential migratory factor is placed on the opposite side of the membrane from the cells. However, as the concentrations of the angiogenic factor can quickly normalise between the upper and lower chambers, it can be challenging to determine if the angiogenesis is due to directed migration or not. A more useful way of establishing a model of directed migration would be to use the under-agarose assay. In this assay a layer of agarose gel is prepared and then wells are punched into the gel, one contained the cells/follicles and the other containing the angiogenic factor (Hoying and Williams, 1996). The endothelial cells from the follicles then

migrate towards the angiogenic factor, allowing directed angiogenesis to be observed.

TSP-1 has a functional role in the induction of follicular atresia as well as its anti-angiogenic activity and as TSP-1 can inhibit angiogenesis and induce follicular atresia it could be a potential therapeutic for PCOS. PCOS is the most common endocrine disorder in women of reproductive age and is characterised by increased angiogenesis and decreased follicular atresia (Agrawal et al., 1998b, Franks et al., 2000) so the use of TSP-1 as a therapeutic for this disorder is an exciting possibility. To establish whether TSP-1 could be of benefit in treating PCOS further studies should be carried out using a model of PCOS. There are several animal models of PCOS available (Beloosesky et al., 2004, Abbott DH et al., 2006, Dumesic et al., 2007) and use of these models would enable investigation into the mechanisms of follicular development in PCOS and establish whether TSP-1 can inhibit follicular angiogenesis and induce follicular atresia in the PCOS ovary, as well as in normal ovaries. A successful outcome of these experiments would confirm the findings of this thesis and would strongly suggest that TSP-1 could be a useful treatment for PCOS.

There is a prenatally androgenised rat model of PCOS available (McNeilly *et al.* unpublished data) that could be utilised to determine the potential of ABT-898 in treating PCOS. First the expression of TSP-1 mRNA and protein would be quantified in the rat model of PCOS as it is anticipated that TSP-1 expression will be decreased in the PCOS ovary. To investigate the potential of TSP-1 as a therapeutic for PCOS,

PCOS rats will be treated with a short course of ABT-898 and the effects on ovarian morphology, angiogenesis, follicular growth and cell death will be determined. It is anticipated that treatment will inhibit angiogenesis and promote atresia of accumulated antral follicles. A successful outcome will be followed by a study in which PCOS rats will be treated with TSP-1 peptide and vaginal smears taken to assess recovery of oestrous cyclicity followed by housing with males to confirm fertility. If successful, the sheep model of PCOS could be utilised to address selected questions and give the data greater relevance to the human.

This thesis has found that Ang-1 up-regulates the expression of pro-survival mediators and inhibits the production of pro-apoptotic factors. To determine whether treatment with Ang-1 could be of benefit in IVM techniques further experiments should be conducted to assess the effect on the expression of factors that regulate follicle health and survival and also to determine whether Ang-1 can affect oocyte health. The culture of oocyte-granulosa cell cultures (OGCs) would determine the effect of Ang-1 treatment on oocyte health and qRT-PCR would enable the change in expression of factors such as pERK, p38, GDF-9 and BMP-15 to be determined. If Ang-1 was found to improve oocyte health and increase expression of factors indicative of follicle health it would support the hypothesis that Ang-1 could be of use in improving follicle survival in culture.

## 8.7 Conclusions

A unique *in vitro* model has been developed to investigate the regulation of follicular angiogenesis in the rodent and to investigate the effects of various factors involved in the angiogenic cascade. Using this model, as well as an *in vivo* marmoset model, the studies reported in this thesis provide compelling evidence that TSP-1 both inhibits follicular angiogenesis and has an extravascular role inducing apoptosis within the ovary. This model has also been utilised to clarify the role of the angiopoietins in the ovary and have shown that, dependent on the dosage used, Ang-1 can both promote follicle survival and also induce follicular atresia. The potential to inhibit angiogenesis in the female reproductive tract and also to improve follicle survival and health has important implications for clinical practice, both in providing potential treatments for clinical conditions and in the improvement of *in vitro* culture techniques.



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## Appendix 1

A selection of the work from chapters 3, 4 and 5 has been published in two papers in Endocrinology, and they have been included in Appendix 1 of this thesis.

Garside, S.A., Harlow, C.R., *et al.* (2010). “Thrombospondin-1 Inhibits Angiogenesis and Promotes Follicular Atresia in a Novel in Vitro Angiogenesis Assay.” *Endocrinology* **151** (3): 1280-1289

Garside, S.A., Henkin, J., *et al.* (2010). “A Thrombospondin-Mimetic Peptide, ABT-898, Suppresses Angiogenesis and Promotes Follicular Atresia in Pre- and Early-Antral Follicles *in vivo*.” *Endocrinology* **151** (12): 5905-5915